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(54) Title: NOVEL CYTOKINE

(57) Abstract

There is disclosed a polypeptide (CD40-L) and DNA sequences, vectors and transformed host cells useful in providing CD40-L polypeptides. More particularly, this invention provides isolated human and murine CD40-L polypeptides that bind to the extracellular binding region of a CD40 receptor.

TITLE

NOVEL CYTOKINE

5

TECHNICAL FIELD OF THE INVENTION

The present invention relates to a novel cytokine. More specifically, the present invention relates to the cloning of a murine and a human cytokine that binds to a human CD40 having both agonist and antagonist activity in soluble and membrane-bound

10   forms.

BACKGROUND OF THE INVENTION

Cytokines that have an "Interleukin" designation are those protein factors that influence immune effector cells. Cytokines designated interleukin-1 through interleukin-12 have been reported and named as an interleukin. Other known cytokines

15   include tumor necrosis factor (TNF), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), mast cell growth factor (MGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF), erythropoietin (EPO), γ-interferon (γ-IFN) and others.

DNAs for two different TNF receptors (Type I and Type II) have been cloned

20   (Smith et al., *Science* 248:1019, 1990; and Schall et al., *Cell* 61:361, 1990). Both forms of TNF receptor are related to each other and belong to a family of receptors whose members include nerve growth factor receptor (Johnson et al., *Cell* 47:545, 1986), B cell antigen CD40 (Stamenkovic et al., *EMBO J.* 8:1403, 1989), T-cell antigen OX40 (Mallett et al., *EMBO J.* 9:1063, 1990), human *Fas* antigen (Itoh et al.,

25   *Cell* 66:233, 1991) and murine 4-1BB receptor (Kwon et al., *Cell. Immunol.* 121:414, 1989 [Kwon et al. I] and Kwon et al., *Proc. Natl. Acad. Sci. USA* 86:1963, 1989 [Kwon et al. II]).

Human CD40 protein (CD40) is a peptide of 277 amino acids having a

30   molecular weight of 30,600, and a 19 amino acid secretory signal peptide comprising predominantly hydrophobic amino acids (Stamenkovic et al.). The molecular weight (exclusive of glycosylation) of the mature human CD40 protein is 28,300. A cDNA encoding human CD40 was isolated from a cDNA library prepared from Burkitt lymphoma cell line Raji. The putative protein encoded by the CD40 cDNA contains a

35   putative leader sequence, trans-membrane domain and a number of other features common to membrane-bound receptor proteins. CD40 has been found to be expressed on B lymphocytes, epithelial cells and some carcinoma cell lines.

A monoclonal antibody (mAb) directed against CD40 has been shown to mediate various functional effects of human B cells. These effects include: (a) homotypic adhesions (Gordon et al., *J. Immunol.* 140:1425, 1988 [Gordon et al. I]); (b) increased cell size (Gordon et al. I and Valle et al., *Eur. J. Immunol.* 19:1463, 1989); (c) proliferation of B cells activated with anti-IgM, anti-CD20 mAb, phorbol ester alone (Clark et al., *Proc. Natl. Acad. Sci. USA* 83:4494, 1986; and Paulie et al., *J. Immunol.* 142:590, 1989), or phorbol ester combined with interleukin-4 (Gordon et al., *Eur. J. Immunol.* 17:1535, 1987 [Gordon et al. II]; and (d) production of IgE (Jabara et al., *J. Exp. Med.* 172:1861, 1990; Zhang et al., *J. Immunol.* 146:1836, 1991) and IgM (Gascan et al., *J. Immunol.* 147:8, 1991) from interleukin-4 (IL-4) stimulated T-depleted cultures.

One such antibody, called mAb 89 by Banchereau et al., *Clin. Immunol. Spectrum* 3:8, 1991 [Banchereau et al. I], was found to induce human B cell proliferation at a relatively low antibody concentration (30 ng/ml or about 10^{-10} M). Proliferation lasted two to three weeks and resulted in a ten-fold expansion of the human B cell population. Optimal stimulation of the B cells occurred when CD40 surface molecule was cross-linked by IgM. Fab fragments of another anti-CD40 mAb induced only a weak proliferative response. Further, Banchereau et al., *Science* 251:70, 1991 [Banchereau et al. II] reported that resting human B cells entered a state of sustained proliferation when incubated with both a murine fibroblastic Ltk⁻ cell line that was transfected with human Fc receptor and with a monoclonal antibody specific for human CD40. Banchereau et al. II found that cross-linking CD40 is necessary for clonal expansion of B cells.

CD23 is a low affinity IgE receptor that has been found to be expressed on most IgM⁻/IgD⁻ mature B cells, but not T cells. CD23 has been sequenced and its sequence was described in Kikutani et al., *Cell* 47:657, 1986. Soluble CD23 (sCD23) was found to induce a pyrogenic reaction in rabbits and this reaction was abrogated by administration of human IgE (Ghaderi et al., *Immunology* 73:510, 1991). Therefore, CD23 may be an appropriate marker for soluble CD40 or CD40-L effects.

Prior to the present invention, a ligand for CD40 was unknown. Accordingly, there is a need in the art to identify and characterize a CD40 ligand (CD40-L).

SUMMARY OF THE INVENTION

A novel cytokine, hereafter referred to as "CD40-L," has been isolated and characterized. The nucleotide sequence and deduced amino acid sequence of representative murine CD40-L cDNA is disclosed in SEQ ID NO:1 and Figure 1, and the amino acid sequence is also listed in SEQ ID NO:2. The nucleotide sequence and

deduced amino acid sequence of representative human CD40-L cDNA is disclosed in SEQ ID NO:11 and Figure 2, and the amino acid sequence is also listed in SEQ ID NO:12. The present invention further comprises other CD40-L polypeptides encoded by nucleotide sequences that hybridize, under moderate or severe stringency

5   conditions, to probes defined by SEQ ID NO:11 (the coding region of human CD40-L), fragments of the sequence extending from nucleotide 46 to nucleotide 828 of SEQ ID NO:11, or to DNA or RNA sequences complementary to Figure 2 (SEQ ID NO:11) or fragments thereof. The invention further comprises nucleic acid sequences which, due to the degeneracy of the genetic code, encode polypeptides substantially identical or

10   substantially similar to polypeptides encoded by the nucleic acid sequences described above, and sequences complementary to them.

CD40-L is a type II membrane polypeptide having an extracellular region at its C-terminus, a transmembrane region and an intracellular region at its N-terminus. A soluble version of murine CD40-L has been found in supernatants from EL-4 cells and

15   EL-4 cells sorted on the basis of a biotinylated CD40/Fc fusion protein described herein. Soluble CD40-L comprises an extracellular region of CD40-L or a fragment thereof. The protein sequence of murine CD40-L is described in Figure 1 and SEQ ID NO:2, and human CD40-L in Figure 2 and SEQ ID NO:12. The extracellular region of murine CD40-L extends from amino acid 47 to amino acid 260 in Figure 1 and SEQ ID

20   NO:2, and of human CD40-L from amino acid 47 to amino acid 261 in Figure 2 and SEQ ID NO:12. CD40-L biological activity is mediated by binding of this cytokine with CD40 and includes B cell proliferation and induction of antibody secretion, including IgE secretion.

The present invention further provides antisense or sense oligonucleotides

25   (deoxyribonucleotides or ribonucleotides) that correspond to a sequence of at least about 12 nucleotides selected from the nucleotide sequence of CD40-L or DNA or RNA sequences complementary to the nucleotide sequence of CD40-L as described in SEQ ID NO:1 and SEQ ID NO:11 and in Figures 1 and 2. Such antisense or sense oligonucleotides prevent transcription or translation of CD40-L mRNA or polypeptides.

30   Further still, the present invention provides CD40-L peptide fragments that correspond to a protein sequence of at least 10 amino acids selected from the amino acid sequence encoded by SEQ ID NO:1 or SEQ ID NO:11 that can act as immunogens to generate antibodies specific to the CD40-L immunogens. Such CD40-L immunogen fragments can serve as antigenic determinants in providing monoclonal antibodies

35   specific for CD40-L.

The invention also provides a human CD40/Fc fusion protein and a soluble CD40 protein (sCD40) comprising the extracellular region of human CD40. Both

sCD40 and CD40/Fc fusion protein can inhibit CD40-L or anti-CD40 mAb induced B cell stimulation, IL-4-induced IgE stimulation and IL-4 induced CD23 induction in B cells.

BRIEF DESCRIPTION OF THE DRAWINGS

5   Figure 1 illustrates nucleotide and amino acid sequences corresponding to murine CD40-L. This protein is a type II polypeptide having its N-terminus as its intracellular domain, followed by a transmembrane region, and an extracellular domain at the C-terminus of the polypeptide. The extracellular domain, which is longer than either the intracellular domain or the transmembrane region, contains one potential N-10   linked glycosylation site and two potential disulfide bonds in view of four cysteine (Cys) residues.

Figure 2 illustrates nucleotide and amino acid sequences corresponding to human CD40-L. This protein is a type II polypeptide having its N-terminus as its intracellular domain, followed by a transmembrane region, and an extracellular domain 15   at the C-terminus of the polypeptide. The extracellular domain, which is longer than either the intracellular domain or the transmembrane region, contains 1 potential N-linked glycosylation site and 2 potential disulfide bonds in view of 5 cysteine (Cys) residues.

Figure 3 illustrates a comparison of protein sequences of human and murine 20   CD40-L showing 77.7% homology at the amino acid level.

Figure 4 illustrates proliferation of T cell depleted human peripheral blood mononuclear cells (PBMC) caused by incubation with CV1 cells transfected with full length murine CD40-L cDNA (SEQ ID NO:1) and expressing bound CD40-L (CD40-L+CV1 cells) when compared with CV1 cells transfected with empty vector (HAVEO) 25   and not expressing bound murine CD40-L. The day 7 proliferation results show that CD40-L+CV1 cells significantly increase proliferation of T- cell depleted PBMC in the presence or absence of interleukin-4 (IL-4).

Figure 5 illustrates a second determination of T cell depleted PBMC 30   proliferation with addition of bound murine CD40-L and 10 ng/ml of IL-4. These data show no co-mitogenic effect of IL-4 but continued strong mitogenic effect of bound CD40-L.

Figure 6 illustrates that bound CD40-L augments IgE secretion.

Figure 7 illustrates that membrane-bound CD40-L stimulates CD23 shedding in 35   the presence of IL-4.

Figure 8 illustrates proliferation of murine splenic B cells caused by membrane-bound murine CD40-L or 7A1 cells, which is a helper T cell clone.

Figure 9 illustrates a comparison of murine EL40.9 cells, a sorted cell line that was sorted on the basis of expression of murine CD40-L and T cells 7A1 for induction of an antigen-specific response indicated by plaque forming cells (PFC) by anti-sheep red blood cells (SCBC).

5   Figure 10 illustrates a comparison of B cell proliferative activity of membrane-bound CD40-L and other cell types transfected with different cDNAs. Membrane-bound CD40-L showed significantly more B cell proliferative activity than a helper T cell clone or other control cells.

Figure 11 illustrates that 7C2 cells (a helper T cell clone) and CV1 cells

10   transfected with murine CD40-L cDNA induce anti SRBC plaque forming cells.

Figure 12 illustrates a comparison of two helper T cell clones with cells expressing membrane-bound CD40-L for inducing murine B cell proliferation.

Figure 13 illustrates induction of antigen-specific plaque forming cells by membrane-bound CD40-L and a helper T cell clone in the presence or absence of added

15   interleukin-2 (IL-2).

Figure 14 shows effects of membrane-bound CD40-L stimulating B cell proliferation and IgE secretion. The effects of membrane-bound CD40-L were inhibited by CD40 receptor but not by TNF receptor.

20   ## DETAILED DESCRIPTION OF THE INVENTION

Novel polypeptides that can act as a ligand for murine and human CD40 have been isolated and sequenced. More particularly, cDNAs encoding these ligands have been cloned and sequenced. Further provided are methods for expression of recombinant CD40-L polypeptides. CD40-L polypeptide include other forms of

25   mammalian CD40-L, such as derivatives or analogs of human or murine CD40-L. Murine and human CD40-L comprise a 214 and 215, respectively amino acid extracellular region at the C-terminus of full length, membrane-bound polypeptide. The extracellular region contains the domain that binds to CD40. Murine and human CD40-L further comprise a homologous hydrophobic 24 amino acid transmembrane region

30   delineated by charged amino acids on either side and a 22 amino acid intracellular region at their N-termini. The present invention further comprises full length CD40-L polypeptides or fragments thereof comprising all or part of the extracellular region or derivatives of the extracellular region and mammalian cells transfected with a cDNA encoding murine or human CD40-L and expressing human or murine CD40-L as a

35   membrane-bound protein.

The present invention comprises isolated DNA sequences encoding CD40-L polypeptides and DNA or RNA sequences complementary to such isolated DNA

sequences. The isolated DNA sequences and their complements are selected from the group consisting of (a) nucleotides 184 through 828, nucleotides 193 through 828 or nucleotides 193 through 762 of the DNA sequence set forth in Figure 2 (SEQ ID NO:11) and their complements, (b) DNA sequences which hybridize to the DNA

5   sequences of (a) or their complements under conditions of moderate stringency and which encode a CD40-L polypeptide, analogs or derivatives thereof, and (c) DNA sequences which, due to the degeneracy of the genetic code, encode CD40-L polypeptides encoded by any of the foregoing DNA sequences and their complements. In addition, the present invention includes vectors comprising DNA sequences

10   encoding CD40-L polypeptides and analogs, and host cells transfected with such vectors.

The novel cytokine disclosed herein is a ligand for CD40, a receptor that is a member of the TNF receptor super family. Therefore, CD40-L is likely to be responsible for transducing signal via CD40, which is known to be expressed, for

15   example, by B lymphocytes. Full-length CD40-L is a membrane-bound polypeptide with an extracellular region at its C terminus, a transmembrane region, and an intracellular region at its N-terminus. A soluble version of CD40-L can be made from the extracellular region or a fragment thereof and a soluble CD40-L has been found in culture supernatants from cells that express a membrane-bound version of CD40-L.

20   The protein sequence of the extracellular region of murine CD40-L extends from amino acid 47 to amino acid 260 in Figure 1 and SEQ ID NO:2. The protein sequence of the extracellular region of human CD40-L extends from amino acid 47 to amino acid 261 in Figure 2 and SEQ ID NO:12. The biological activity of CD40-L is mediated by binding to CD40 or a species-specific homolog thereof and comprises proliferation of B cells

25   and induction of immunoglobulin secretion from activated B cells. CD40-L (including soluble monomeric and oligomeric forms, as well as membrane-bound forms) can effect B cell proliferation and immunoglobulin secretion (except IgE secretion) without the presence of added IL-4, in contrast to anti-CD40 antibodies, which require IL-4 and cross-linking to mediate activity.

30   CD40-L refers to a genus of polypeptides which are capable of binding CD40, or mammalian homologs of CD40. As used herein, the term "CD40-L" includes soluble CD40-L polypeptides lacking transmembrane and intracellular regions, mammalian homologs of human CD40-L, analogs of human or murine CD40-L or derivatives of human or murine CD40-L.

35   CD40-L may also be obtained by mutations of nucleotide sequences coding for a CD40-L polypeptide. A CD40-L analog, as referred to herein, is a polypeptide substantially homologous to a sequence of human or murine CD40-L but which has an

amino acid sequence different from native sequence CD40-L (human or murine species) polypeptide because of one or a plurality of deletions, insertions or substitutions. Analogs of CD40-L can be synthesized from DNA constructs prepared by oligonucleotide synthesis and ligation or by site-specific mutagenesis techniques.

5   The primary amino acid structure of human or murine CD40-L may be modified to create CD40-L derivatives by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like, or by creating amino acid sequence mutants. Covalent derivatives of CD40-L are prepared by linking particular functional groups to CD40-L amino acid side chains or at 10 the N-terminus or C-terminus of a CD40-L polypeptide or the extracellular domain thereof. Other derivatives of CD40-L within the scope of this invention include covalent or aggregative conjugates of CD40-L or its fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugate may comprise a signal or leader polypeptide 15 sequence at the N-terminal region or C-terminal region of a CD40-L polypeptide which co-translationally or post-translationally directs transfer of the conjugate from its site of synthesis to a site inside or outside of the cell membrane or cell wall (e.g. the α-factor leader of *Saccharomyces*). CD40-L polypeptide fusions can comprise polypeptides added to facilitate purification and identification of CD40-L (e.g. poly-His), or fusions 20 with other cytokines to provide novel polyfunctional entities. Other cytokines include, for example, any of interleukins-1 through 13, TNF (tumor necrosis factor), GM-CSF (granulocyte macrophage-colony stimulating factor), G-CSF (granulocyte-colony stimulating factor), MGF (mast cell growth factor), EGF (epidermal growth factor), PDGF (platelet-derived growth factor), NGF (nerve growth factor), EPO 25 (erythropoietin), γ-IFN (gamma interferon), 4-1BB-L (4-1BB ligand) and other cytokines that affect immune cell growth, differentiation or function.

         Nucleic acid sequences within the scope of the present invention include DNA and/or RNA sequences that hybridize to the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:11 or the complementary strands, under conditions of moderate or severe 30 stringency. Moderate stringency hybridization conditions refer to conditions described in, for example, Sambrook et al. *Molecular Cloning: A Laboratory Manual,* 2 ed. Vol. 1, pp. 1.101-104, Cold Spring Harbor Laboratory Press, (1989). Conditions of moderate stringency, as defined by Sambrook et al., include use of a prewashing solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0) and hybridization 35 conditions of 50°C, 5 X SSC, overnight. Conditions of severe stringency include higher temperatures of hybridization and washing.

Biological activity of CD40-L may be determined, for example, by competition for binding to the ligand binding domain of CD40 (i.e. competitive binding assays). Both murine CD40-L and human CD40-L bind to human CD40. The binding affinity of murine CD40-L (expressed on sorted murine EL-40.9 cells) for human CD40 was

5   approximately
$1.74 \times 10^9 M^{-1}$. Similarly, the binding affinity of murine CD40-L (expressed on unsorted murine EL-46.1 cells) for human CD40 was approximately $2.3 \times 10^9 M^{-1}$. Both binding affinity measurements are within a range typical of cytokine/cytokine receptor binding.

10   One configuration of a competitive binding assay for CD40-L polypeptide uses a radiolabeled, soluble murine CD40-L according to Figure 1 (SEQ ID NO:1) or human CD40-L according to Figure 2 (SEQ ID NO:11), and intact cells expressing CD40 (e.g., human B cells). Instead of intact cells, one could substitute soluble CD40 (such as a CD40/Fc fusion protein) bound to a solid phase through a Protein A or Protein G

15   interaction with the Fc region of the fusion protein. A second configuration of a competitive binding assay utilizes radiolabeled soluble CD40 such as a CD40/Fc fusion protein, and intact cells expressing CD40-L. Alternatively, soluble CD40-L could be bound to a solid phase.

Competitive binding assays can be performed using standard methodology.

20   For example, radiolabeled murine CD40-L can be used to compete with a putative CD40-L homolog to assay for binding activity against surface-bound CD40. Qualitative results can be obtained by competitive autoradiographic plate binding assays, or Scatchard plots may be utilized to generate quantitative results.

Competitive binding assays with intact cells expressing CD40 can be performed

25   by two methods. In a first method, B cells are grown either in suspension or by adherence to tissue culture plates. Adherent cells can be removed by treatment with 5 mM EDTA treatment for ten minutes at 37° C. In a second method, transfected COS cells expressing membrane-bound CD40 can be used. COS cells or another mammalian cell can be transfected with human CD40 cDNA in an appropriate vector to

30   express full length CD40 with an extracellular region exterior to the cell.

Alternatively, soluble CD40 can be bound to a solid phase such as a column chromatography matrix, or a tube or similar substrate suitable for analysis for the presence of a detectable moiety such as ^{125}I. Binding to a solid phase can be accomplished, for example, by obtaining a CD40/Fc fusion protein and binding it to a

35   protein A or protein G surface.

Another means to measure the biological activity of CD40-L and homologs thereof is to utilize conjugated, soluble CD40 (for example, ^{125}I-CD40/Fc) in

competition assays similar to those described above. In this case, however, intact cells expressing CD40-L, or soluble CD40-L bound to a solid substrate, are used to measure competition for binding of conjugated, soluble CD40 to CD40-L by a sample containing a putative CD40 homolog.

5   CD40-L may also be assayed by measuring biological activity in a B cell proliferation assay. Human B cells may be obtained from human tonsils by purification by negative selection and Percoll density sedimentation, as described by Defrance et al., *J. Immunol.* 139:1135, 1987. Burkitt lymphoma cell lines may be used to measure cell proliferation in response to CD40-L. Examples of Burkitt lymphoma cell lines include,

10   for example, Raji (ATCC CCL 86), Daudi (ATCC CCL 213) and Namalwa (ATCC CRL 1432). Membrane-bound CD40-L stimulated B cell proliferation. Oligomeric, preferably dimeric, CD40-L can stimulate B cell proliferation. CD40 (receptor) antagonizes CD40-L proliferation of B cells.

Yet another assay for determining CD40-L biological activity is to measure

15   immunoglobulin produced by B cells in response to activation by CD40-L or a derivative or analog thereof. Polyclonal immunoglobulin secretion can be measured, for example, by incubating with 5×10^5 B cells/ml in culture for at least seven days. Immunoglobulin (Ig) production can be measured by an ELISA assay such as one described in Maliszewski et al., *J. Immunol.* 144:3028, 1990 [Maliszewski et al. I] or

20   Maliszewski et al., *Eur J. Immunol.* 20:1735, 1990 [Maliszewski et al. II]. Murine B cells can be obtained, for example, from mice and cultured according to procedures described in Grabstein et al., *J. Exp. Med.* 163:1405, 1986 [Grabstein et al. I], Maliszewski et al. I, and Maliszewski et al. II.

CD40-L can be used in a binding assay to detect cells expressing CD40. For

25   example, murine CD40-L according to Figure 1 (SEQ ID NO:1) or human CD40-L according to Figure 2 (SEQ ID NO:11), or an extracellular domain or a fragment thereof, can be conjugated to a detectable moiety such as ^{125}I. Radiolabeling with ^{125}I can be performed by any of several standard methodologies that yield a functional ^{125}I-CD40-L molecule labeled to high specific activity. Alternatively, another detectable

30   moiety such as an enzyme that can catalyze a colorimetric or fluorometric reaction, biotin or avidin may be used. Cells expressing CD40 can be contacted with conjugated CD40-L. After incubation, unbound conjugated CD40-L is removed and binding is measured using the detectable moiety.

CD40-L polypeptides may exist as oligomers, such as dimers or trimers.

35   Oligomers are linked by disulfide bonds formed between cysteine residues on different CD40-L polypeptides. Alternatively, one can link two soluble CD40-L domains with a Gly4SerGly5Ser linker sequence, or other linker sequence described in United States

Patent 5,073,627, which is incorporated by reference herein. CD40-L polypeptides may also be created by fusion of the C terminal of soluble CD40-L (extracellular domain) to the Fc region of IgG1 (for example, SEQ ID NO:3) as described for the CD40/Fc fusion protein. CD40-L/Fc fusion proteins are allowed to assemble much like

5   heavy chains of an antibody molecule to form divalent CD40-L. If fusion proteins are made with both heavy and light chains of an antibody, it is possible to form a CD40-L oligomer with as many as four CD40-L extracellular regions.

Fusion proteins can be prepared using conventional techniques of enzyme cutting and ligation of fragments from desired sequences. PCR techniques employing

10   synthetic oligonucleotides may be used to prepare and/or amplify the desired fragments. Overlapping synthetic oligonucleotides representing the desired sequences can also be used to prepare DNA constructs encoding fusion proteins. Fusion proteins can also comprise CD40-L and two or more additional sequences, including a leader (or signal peptide) sequence, Fc region, linker sequence, and sequences encoding highly

15   antigenic moieties that provide a means for facile purification or rapid detection of a fusion protein.

Signal peptides facilitate secretion of proteins from cells. An exemplary signal peptide is the amino terminal 25 amino acids of the leader sequence of human interleukin-7 (IL-7; Goodwin et al., *Proc. Natl. Acad. Sci. U.S.A.* 86:302, 1989;

20   Figure 2B). Other signal peptides may also be employed. For example, certain nucleotides in the IL-7 leader sequence can be altered without altering the amino acid sequence. Additionally, amino acid changes that do not affect the ability of the IL-7 sequence to act as a leader sequence can be made.

The Flag® octapeptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) does not alter the

25   biological activity of fusion proteins, is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid detection and facile purification of the expressed fusion protein. The Flag® sequence is also specifically cleaved by bovine mucosal enterokinase at the residue immediately following the Asp-Lys pairing, fusion proteins capped with this peptide may also be resistant to

30   intracellular degradation in *E. coli*. A murine monoclonal antibody that binds the Flag® sequence has been deposited with the ATCC under accession number HB 9259; methods of using the antibody in purification of fusion proteins comprising the Flag® sequence are described in U.S. Patent 5,011,912, which is incorporated by reference herein.

35   Suitable Fc regions are defined as Fc regions that can bind to protein A or protein G, or alternatively, are recognized by an antibody that can be used in purification or detection of a fusion protein comprising the Fc region. Preferable Fc

regions include the Fc region of human IgG_1 or murine IgG_1. One example is the human IgG_1 Fc region shown in SEQ ID NO:3; another example is an Fc region encoded by cDNA obtained by PCR from oligonucleotide primers from SEQ ID NO:9 and SEQ ID NO:10 with human cDNA as a template. Portions of a suitable Fc region

5   may also be used, for example, an Fc region of human IgG_1 from which has been deleted a sequence of amino acids responsible for binding to protein A, such that the resultant Fc region binds to protein G but not protein A.

The $[Gly_4Ser]_3$ repeat sequence provides a linker sequence that separates the extracellular region of the CD40-L from the Fc portion of the fusion protein by a

10   distance sufficient to ensure that the CD40-L properly folds into its secondary and tertiary structures. Suitable linker sequences (1) will adopt a flexible extended conformation, (2) will not exhibit a propensity for developing an ordered secondary structure which could interact with the functional domains of fusion proteins; and (3) will have minimal hydrophobic or charged character which could promote interaction

15   with the functional protein domains. Typical surface amino acids in flexible protein regions include Gly, Asn and Ser. Virtually any permutation of amino acid sequences containing Gly, Asn and Ser would be expected to satisfy the above criteria for a linker sequence. Other near neutral amino acids, such as Thr and Ala, may also be used in the linker sequence. The length of the linker sequence may vary without significantly

20   affecting the biological activity of the fusion protein. Linker sequences are unnecessary where the proteins being fused have non-essential N- or C-terminal amino acid regions which can be used to separate the functional domains and prevent steric interference.

CD40-L polypeptides may exist as soluble polypeptides comprising the extracellular domain of CD40-L as shown in Figure 1 (SEQ ID NO:1) and Figure 2

25   (SEQ ID NO:11) or as membrane-bound polypeptides comprising the extracellular domain, a transmembrane region and a short intracellular domain, as shown in Figure 1 (SEQ ID NO:1) and Figure 2 (SEQ ID NO:11) for the murine and human sequences, respectively. Moreover, the present invention comprises oligomers of CD40-L extracellular domains or fragments thereof, linked by disulfide interactions, or

30   expressed as fusion polymers with or without spacer amino acid linking groups. For example, a dimer CD40-L molecule can be linked by an IgG Fc region linking group.

Without being bound by theory, membrane-bound CD40-L and oligomeric CD40-L can achieve activity stimulating Ig formation and proliferation of B cells previously only achieved by cross-linked anti-CD40 antibody in the presence of IL-4.

35   It further appears likely that monomeric soluble CD40-L, comprising only the extracellular domain of CD40-L and capable of binding to CD40 receptor, will serve to antagonize the activity of membrane-bound and oligomeric CD40-L and/or cross-linked

anti-CD40 antibodies. It further appears likely that the interaction of membrane-bound CD40-L with CD40 is the principal molecular interaction responsible for T cell contact dependent induction of B cell growth and differentiation to both antigen specific antibody production and polyclonal Ig secretion. In this regard, a mammalian cell

5   transfected with a cDNA encoding full length CD40-L (i.e., being membrane-bound and having an intracellular domain, a transmembrane region and an extracellular domain or a fragment thereof) can mimic T cells in their ability to induce B cell growth, differentiation and stimulation of antigen-specific antibody production. It appears that activities of oligomeric soluble CD40-L, preferably a dimer of extracellular regions, can

10   mimic the biological activities of membrane-bound CD40-L. Moreover, soluble monomeric CD40-L (comprising the extracellular domain or a fragment thereof) can bind to CD40 receptor to prevent T cell interaction with B cells and therefor have activity similar to CD40 (receptor) extracellular domain which itself may be in monomeric or in oligomeric form. Alternatively, CD40-L can be oligomeric (preferably

15   a dimer) to act as a soluble factor capable of inducing B cell growth, differentiation and stimulation of antigen-specific antibody production. Accordingly, it appears that membrane-bound CD40-L and oligomeric CD40-L act as CD40 agonists, while soluble (monomeric) CD40-L and soluble CD40 act as CD40 antagonists by blocking CD40 receptor sites without significantly transducing signal or by preventing CD40-L binding

20   to CD40 sites on B cells and other target cells.

        Both CD40 agonists and CD40 antagonists will have useful therapeutic activity. For example, CD40 agonists (i.e., membrane-bound CD40-L and oligomeric CD40-L) are useful as vaccine adjuvants and for stimulating mAb production from hybridoma cells. CD40 antagonists (i.e., CD40 receptor, CD40/Fc and possibly soluble,

25   monomeric CD40-L) are useful for treating autoimmune diseases characterized by presence of high levels of antigen-antibody complexes, such as allergy, lupus, rheumatoid arthritis, insulin dependent diabetes mellitus (IDDM), graft versus host disease (GVHD) and others.

        IgE secretion from human B cells can be induced by IL-4 in the presence of T

30   cells (Vercelli et al., *J. Exp. Med.* 169:1295, 1989). Further, IgE production can be induced from T cell depleted PBM (peripheral blood mononuclear cells) by addition of an anti-CD40 mAb (Jabara et al., *J. Exp. Med.* 172:1861, 1990 and Zhang et al., *J. Immunol.* 146:1836, 1991). The present invention further includes a method for inhibiting IgE production from activated B cells, activated by IL-4 in the presence of T

35   cells or by CD40-L (preferably, membrane-bound CD40-L), comprising administering an effective amount of a CD40/Fc fusion protein, as described herein, or a soluble CD40 encoded by the cDNA sequence described in SEQ ID NO. 3. Similarly, CD40

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receptors and possibly soluble CD40-L (monomer only) can also block secretion of other antibody isotypes.

The present invention further includes CD40-L polypeptides with or without associated native-pattern glycosylation. CD40-L expressed in yeast or mammalian expression systems (e.g., COS-7 cells) may be similar to or significantly different from a native CD40-L polypeptide in molecular weight and glycosylation pattern, depending upon the choice of expression system. Expression of CD40-L polypeptides in bacterial expression systems, such as *E. coli*, provides non-glycosylated molecules.

DNA constructs that encode various additions or substitutions of amino acid residues or sequences, or deletions of terminal or internal residues or sequences not needed for biological activity or binding can be prepared. For example, the extracellular CD40-L N-glycosylation site can be modified to preclude glycosylation while allowing expression of a homogeneous, reduced carbohydrate analog using yeast expression systems. N-glycosylation sites in eukaryotic polypeptides are characterized by an amino acid triplet Asn-X-Y, wherein X is any amino acid except Pro and Y is Ser or Thr. Appropriate modifications to the nucleotide sequence encoding this triplet will result in substitutions, additions or deletions that prevent attachment of carbohydrate residues at the Asn side chain. In another example, sequences encoding Cys residues can be altered to cause the Cys residues to be deleted or replaced with other amino acids, preventing formation of incorrect intramolecular disulfide bridges upon renaturation. Human CD40-L comprises five Cys residues in its extracellular domain. Thus, at least one of the five Cys residues can be replaced with another amino acid or deleted without effecting protein tertiary structure or disulfide bond formation.

Other approaches to mutagenesis involve modification of sequences encoding dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present. Sub-units of a CD40-L polypeptide may be constructed by deleting sequences encoding terminal or internal residues or sequences.

CD40-L polypeptides are encoded by multi-exon genes. The present invention further includes alternative mRNA constructs which can be attributed to different mRNA splicing events following transcription and which share regions of identity or similarity with the cDNAs disclosed herein.

Antisense or sense oligonucleotides comprise a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to target CD40-L mRNA (sense) or CD40-L DNA (antisense) sequences. Antisense or sense oligonucleotides, according to the present invention, comprise a fragment of SEQ ID NO:1 or SEQ ID NO:11, or a DNA or RNA complement of SEQ ID NO:1 or SEQ ID NO:11. Such a fragment comprises at least about 14 nucleotides. Preferably, such a fragment comprises from

about 14 to about 30 nucleotides. The ability to create an antisense or a sense oligonucleotide, based upon a cDNA sequence for CD40-L is described in, for example, Stein and Cohen, *Cancer Res.* 48:2659, 1988 and van der Krol et al., *BioTechniques* 6:958, 1988.

5   Binding of antisense or sense oligonucleotides to target nucleic acid sequences results in the formation of duplexes that block translation (RNA) or transcription (DNA) by one of several means, including enhanced degradation of the duplexes, premature termination of transcription or translation, or by other means. Suitable polymerase promotors include promotors for any RNA polymerase, or promotors for

10 any DNA polymerase. Antisense or sense oligonucleotides further comprise oligonucleotides having modified sugar-phosphodiester backbones (or other sugar linkages, such as those described in WO91/06629) and wherein such sugar linkages are resistant to endogenous nucleases. Such oligonucleotides with resistant sugar linkages are stable *in vivo* (i.e., capable of resisting enzymatic degradation) but retain sequence

15 specificity to be able to bind to target nucleotide sequences. Other examples of sense or antisense oligonucleotides include those oligonucleotides which are covalently linked to organic moieties, such as those described in WO 90/10448, and other moieties that increases affinity of the oligonucleotide for a target nucleic acid sequence, such as poly-(L-lysine). Further still, intercalating agents, such as ellipticine, and alkylating agents

20 or metal complexes may be attached to sense or antisense oligonucleotides to modify binding specificities of the antisense or sense oligonucleotide for the target nucleotide sequence. Antisense or sense oligonucleotides may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, CaPO$_4$-mediated DNA transfection, electroporation, or other gene transfer vectors such

25 as Epstein-Barr virus. Antisense or sense oligonucleotides are preferably introduced into a cell containing the target nucleic acid sequence by insertion of the antisense or sense oligonucleotide into a suitable retroviral vector, then contacting the cell with the retrovirus vector containing the inserted sequence, either *in vivo* or *ex vivo*. Suitable retroviral vectors include, but are not limited to, the murine retrovirus M-MuLV, N2 (a

30 retrovirus derived from M-MuLV), or or the double copy vectors designated DCT5A, DCT5B and DCT5C (see PCT Application US 90/02656). Alternatively, other promotor sequences may be used to express the oligonucleotide.

Sense or antisense oligonucleotides may also be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand

35 binding molecule, as described in WO 91/04753. Suitable ligand binding molecules include, but are not limited to, cell surface receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. Preferably, conjugation of the

15

ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell.

5   Alternatively, a sense or an antisense oligonucleotide may be introduced into a cell containing the target nucleic acid sequence by formation of an oligonucleotide-lipid complex, as described in WO 90/10448. The sense or antisense oligonucleotide-lipid complex is preferably dissociated within the cell by an endogenous lipase.

       The sequence of murine CD40-L cDNA was obtained by direct expression techniques. The sequence of human CD40-L was obtained by cross-species

10 hybridization techniques using the murine CD40-L cDNA as a probe.

       We cloned murine CD40-L by first obtaining a clone of the extracellular region of human CD40 (the receptor) by polymerase chain reaction (PCR) techniques using primers based upon a sequence published in Stamenkovic et al. (SEQ ID NO:4). An upstream oligonucleotide primer 5'-CCGTCGACCACCATGGTTCGTCTGCC -3'

15 (SEQ ID NO:5) introduces a *Sal* 1 site upstream from an initiator methionine of CD40 and a downstream oligonucleotide primer 5'-CCGTCGACGTCTAGAGCCGATCCTGGGG-3' (SEQ ID NO:6) inserts a termination codon after amino acid 192 of CD40, followed by *Xba* 1 and *Sal* 1 sites. The amplified cDNA was digested with *Sal* 1 and cloned into pDC406 (McMahan et

20 al., *EMBO J.* 10:2821, 1991) to construct pDC406/s CD40.

       A second CD40 receptor fragment (SEQ ID NO:4) was obtained by PCR techniques for fusion to the Fc domain of human IgG1 (SEQ ID NO:3). Briefly, The upstream oligonucleotide primer (SEQ ID NO:5) and fusion template (SEQ ID NO:4) were the same as before. The downstream oligonucleotide primer was 5'-

25 ACAAGATCTGGGCTCTACGTATCTCAGCCGATCCTGGGGAC-3' (SEQ ID NO:7) that inserts amino acids Tyr Val Glu Pro Arg (SEQ ID NO:8) after amino acid 193 of CD40. Glu and Pro are the first two amino acids of a hinge region of human IgG1, and are followed by a *Bgl* II restriction site. The *Bgl* II restriction site was used to fuse the extracellular domain of CD40 to the remainder of human IgG1 Fc region.

30   Other fusion proteins comprising ligand binding domains from other receptors can be made by obtaining a DNA sequence for the ligand binding domain of a receptor and fusing this sequence to a DNA sequence encoding an Fc region of an antibody molecule that binds to protein A or protein G, or another polypeptide that is capable of affinity purification, for example, avidin or streptavidin. The resultant gene construct

35 can be introduced into mammalian cells to transiently express a fusion protein. Receptor/Fc fusion proteins can be purified by protein A or protein G affinity purification. Receptor/avidin fusion proteins can be purified by biotin affinity

chromatography. The fusion protein can later be removed from the column by eluting with a high salt solution or another appropriate buffer.

We obtained a cDNA encoding human IgG1 Fc region by PCR amplification using cDNA from human cells as a template and an upstream oligonucleotide primer 5'-

5   TATTAATCATTCAGTAGGGCCCAGATCTTGTGACAAAACTCAC-3' (SEQ ID NO:9) and a downstream oligonucleotide primer 5'-GCCAGCTTAACTAGTTCATTTACCCGGAGACAGGGAGA-3" (SEQ ID NO:10). The PCR amplified cDNA introduced a *Bgl* II site near the beginning of the hinge region, which was used to ligate CD40 extracellular domain to construct a s CD40/Fc

10   fusion cDNA, which was ligated into pDC406 to construct pDC406/CD40/Fc. Other suitable Fc regions are defined as any region that can bind with high affinity to protein A or protein G, and includes the Fc region of human IgG1 or murine IgG1. One example is the human IgG1 Fc region shown in SEQ ID NO:3 or the cDNA obtained by PCR from oligonucleotide primers from SEQ ID NO:9 and SEQ ID NO:10 with

15   human cDNA as a template.

Receptor/Fc fusion molecules preferably are synthesized in recombinant mammalian cell culture because they are generally too large and complex to be synthesized by prokaryotic expression methods. Examples of suitable mammalian cells for expressing a receptor/Fc fusion protein include CV-1 cells (ATCC CCL 70) and

20   COS-7 cells (ATCC CRL 1651), both derived from monkey kidney.

The DNA construct pDC406/CD40/Fc was transfected into the monkey kidney cell line CV-1/EBNA (ATCC CRL 10478). The pDC406 plasmid includes regulatory sequences derived from SV40, human immunodeficiency virus (HIV), and Epstein-Barr virus (EBV). The CV-1/EBNA cell line was derived by transfection of the CV-1

25   cell line with a gene encoding Epstein-Barr virus nuclear antigen-1 (EBNA-1) and constitutively express EBNA-1 driven from human CMV immediate-early enhancer/promoter. An EBNA-1 gene allows for episomal replication of expression vectors, such as pDC406, that contain the EBV origin of replication.

Transfectants expressing CD40/Fc fusion protein are initially identified using

30   dot blots or Western blots. The supernatants are then subjected to dot blot or gel electrophoresis followed by transfer of the electrophoresed proteins for binding to G28-5 mAb (an antibody that binds to human CD40 receptor). The blotted proteins were then incubated with radiolabeled with ^{125}I-protein A, washed to remove unbound label, and examined for expression of Fc. Monoclonal antibody G28-5 was produced

35   according to Clark et al., *supra.*

Once cells expressing the fusion construct were identified, large scale cultures of transfected cells were grown to accumulate supernatant from cells expressing

CD40/Fc. CD40/Fc fusion protein in supernatant fluid was purified by affinity purification. Briefly, one liter of culture supernatant containing CD40/Fc fusion protein was purified by filtering mammalian cell supernatants (e.g., in a 0.45µ filter) and applying filtrate to a protein A/G antibody affinity column (Schleicher and Schuell,

5   Keene, NH) at 4°C at a flow rate of 80 ml/hr for a 1.5 cm x 12.0 cm column. The column was washed with 0.5 M NaCl in PBS until free protein could not be detected in wash buffer. Finally, the column was washed with PBS. Bound fusion protein was eluted from the column with 25 mM citrate buffer, pH 2.8, and brought to pH 7 with 500 mM Hepes buffer, pH 9.1. Silver-stained SDS gels of the eluted CD40/Fc fusion

10 protein showed it to be >98% pure.

Soluble CD40 (sCD40) and CD40/Fc fusion proteins were made as described herein. The supernatants were purified through a G28-5 (anti-CD40 mAb) affinity column to affinity purify sCD40 expressed by the transfected CV-1/EBNA cells. Protein-containing fractions were pooled and aliquots removed for G28-5 binding

15 assays and analysis by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) in the presence of 1 mM dithiothreitol as a reducing agent. A single band was seen of molecular weight 28,100 daltons. In the absence of a reducing agent, SDS-PAGE analysis of sCD40 revealed two bands, a major band of molecular weight 56,000 and a minor band of molecular weight 28,000. The banding pattern indicates

20 that the majority of sCD40 exists as a disulfide-linked homodimer in solution. The 28,000 band is free monomer.

CD40 proteins were visualized by silver staining. Sample protein concentrations were determined using a micro-BCA assay (Pierce) with ultrapure bovine serum albumin as standard. Soluble CD40 purity and protein concentration

25 were confirmed by amino acid analysis. Purified soluble CD40 was absorbed to PVDF paper and the paper subjected to automated Edman degradation on an Applied Biosystems model 477A protein sequencer according to manufacturers instructions for N-terminal protein sequencing. This procedure checked the protein sequence of sCD40.

30 Soluble CD40 and CD40/Fc fusion protein were able to modulate human B cell responses in the absence of anti-CD40 mAb (G28-5). Purified tonsillar B cells were cultured with anti-IgM and human IL-4 and either sCD40 or CD40/Fc fusion protein was added. Neither form of CD40 had an inhibitory effect on B cell proliferation (as measured by tritiated thymidine incorporation). IL-4 receptor, by contrast, inhibited

35 IL-4-induced B cell proliferation in a concentration-dependent manner.

Soluble CD40 and CD40/Fc were tested for their ability to inhibit IL-4 induced IgE secretion in a 2-donor MLC (mixed lymphocyte culture) system. In three

experiments, the level of IgE production was reduced as the concentration of CD40 was increased. Soluble CD40, added at a concentration of 10 µg/ml, was able to completely inhibit IgE secretion in this model of allergy. Further, CD40/Fc had similar effects as its soluble counterpart. However, addition of an IL-7 receptor-Fc fusion protein (made
5   by similar procedures with a published IL-7 receptor sequence) did not affect secretion of IgE in this model.

    Levels of CD23 were also measured in the same MLC in response to sCD40 or CD40/Fc fusion proteins. Soluble CD40 produced a small, but reproducible decrease in sCD23 level at day 6 compared to cultures stimulated with IL-4 alone, however a stronger inhibitory effect was pronounced at day 12 in the same cultures. Soluble
10   CD23 induction by IL-4-stimulated T-depleted PBM (peripheral blood macrophages) E⁻ cells was similarly affected by addition of sCD40, causing a small decrease in sCD23 levels at day 6 and a more pronounced inhibition at day 12. In each culture system, the results with CD40/Fc fusion protein were substantially the same as with sCD40.

    In an effort to isolate a cDNA for a CD40-L, purified CD40/Fc fusion protein
15   was radioiodinated with ^{125}I using a commercially available solid phase agent (IODO-GEN, Pierce). In this procedure, 5 µg of IODO-GEN were plated at the bottom of a 10 x 75 mm glass tube and incubated for twenty minutes at 4° C with 75 µl of 0.1 M sodium phosphate, pH 7.4 and 20 µl (2 mCi) Na^{125}I. The solution was then transferred to a second glass tube containing 5 µg of CD40/Fc in 45 µl PBS (phosphate
20   buffered saline) and this reaction mixture was incubated for twenty minutes at 4° C. The reaction mixture was fractionated by gel filtration on a 2 ml bed volume of Sephadex® G-25 (Sigma), and then equilibrated in RPMI 1640 medium containing 2.5% (v/v) bovine serum albumin (BSA), 0.2% (v/v) sodium azide and 20 mM Hepes, pH 7.4 binding medium. The final pool of ^{125}I CD40/Fc was diluted to a working
25   stock solution of 1 x 10⁻⁷ M in binding medium and stored for up to one month at 4° C without detectable loss of receptor binding activity.

    A cDNA library was prepared from a EL4 cell line sorted by FACS (fluorescence activated cell sorting) on the basis of binding of a biotinylated CD40/Fc fusion protein. Cells were sorted five times until there was a significant shift in
30   fluorescence intensity based upon expression of a ligand for CD40 by the sorted EL-4 cells. The five-times sorted cells were called EL-40.5 cells and these cells were cultured for the purposes of creating a cDNA library from EL-40.5 mRNA. Briefly, cDNA was synthesized, inserted into empty pDC406 vector and transformed into E. coli. Transformants were pooled, and the DNA from the pools was isolated and
35   transfected into CV1-EBNA cells to create an expression cloning library. Transfected CV1-EBNA cells were cultured on slides for three days to permit transient expression

of CD40-L. The slides containing the transfected cells were then incubated with radioiodinated CD40/Fc, washed to remove unbound CD40/Fc, and fixed with gluteraldehyde. The fixed slides were dipped in liquid photographic emulsion and exposed in the dark. After developing the slides, they were individually examined with

5   a microscope and cells expressing CD40-L were identified by the presence of autoradiographic silver grains against a light background.

The expression cloning library from EL-40.5 cells was screened and one pool, containing approximately 2000 individual clones, was identified as positive for binding ^{125}I labeled CD40/Fc fusion protein. This pool was broken down into smaller pools of

10 approximately 200 colonies. The smaller pools were screened as described above. One of the smaller pools was positive for CD40-L.

A single clone was isolated and sequenced by standard techniques, to provide the cDNA sequence and deduced amino acid sequence of murine CD40-L as shown in Figure 1 and SEQ ID NO:1.

15 The human homolog CD40-L cDNA was found by cross species hybridization techniques. Briefly, a human peripheral blood lymphocyte (PBL) cDNA library was made from peripheral blood lymphocytes treated with OKT3 antibody (ATCC, Rockville MD) that binds to CD3 (10 ng/ml) and interleukin-2 (IL-2, 10 ng/ml) for six days. The PBL cells were washed and then stimulated for 4 hours with 10 ng/ml PMA

20 (phorbol myristate acetate, Sigma St Louis) and 500 ng/ml ionomycin (Calbiochem). Messenger RNA was isolated from stimulated PBL cells, cDNA formed and cDNA was ligated into *Eco* R1 linkers. Ligated cDNA was inserted into the *Eco* R1 site of λgt10 phage cloning vehicle (Gigapak® Stratagene, San Diego, CA) according to manufacturer's instructions. Phage were amplified, plated at densities densities of

25 approximately 20,000 phage per 15 cm plate. and phage lifts were performed, as described in Maniatis et al., *Molecular Biology: A Laboratory Manual*, Cold Spring Harbor Laboratory, NY, 1982 , pages 316-328. A murine probe was constructed corresponding to the coding region of murine CD40-L from nucleotide 13 to nucleotide 793 of SEQ ID NO:1 and Figure 1. This probe was hybridized to to the PBL library

30 phage lifts under conditions of moderate to severe stringency. Briefly, hybridization conditions were 6 X SSC, 1 X Denhardt's solution, 2 mM EDTA, 0.5% Np40 (Nonidet P-40 detergent) at 63 °C overnight. This was followed by washing in 3 X SSC, 0.1% SDS for three hours at 55°C, followed by overnight exposure to X-Ray film. Positive plaques were identified at a frequency of approximately 1 per 1000

35 plaques. Positive plaques were purified twice and cDNA was prepared from amplified cultures.

20

One can utilize the murine or human CD40-L cDNA sequences disclosed herein to obtain cDNAs encoding other mammalian homologs of murine or human CD40-L by cross-species hybridization techniques. Briefly, an oligonucleotide probe is created from the nucleotide sequence of the extracellular region of murine CD40-L as described

5 in Figure 1 (SEQ ID NO:1) or human CD40-L as described in Figure 2 (SEQ ID NO:11). This probe can be made by standard techniques, such as those described in Maniatis et al. *supra.* The murine or human probe is used to screen a mammalian cDNA library or genomic library under moderate stringency conditions. Examples of mammalian cDNA or genomic libraries include, for cDNA, a library made from the

10 mammal's peripheral blood lymphocytes. Alternatively, various cDNA libraries or mRNAs isolated from various cell lines can be screened by Northern hybridization to determine a suitable source of mammalian CD40-L DNA or mRNA.

Recombinant expression vectors for expression of CD40-L by recombinant DNA techniques include a CD40-L DNA sequence comprising a synthetic or cDNA-

15 derived DNA fragment encoding a CD40-L polypeptide, operably linked to a suitable transcriptional or translational regulatory nucleotide sequence, such as one derived from a mammalian, microbial, viral, or insect gene. Examples of regulatory sequences include sequences having a regulatory role in gene expression (e.g., a transcriptional promoter or enhancer), optionally an operator sequence to control transcription, a

20 sequence encoding an mRNA ribosomal binding site, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the CD40-L DNA sequence. Thus, a promoter nucleotide sequence is operably linked to a CD40-L DNA sequence if the promoter nucleotide sequence controls the transcription of the

25 CD40-L DNA sequence. Still further, a ribosome binding site may be operably linked to a sequence for a CD40-L polypeptide if the ribosome binding site is positioned within the vector to encourage translation. In addition, sequences encoding signal peptides can be incorporated into expression vectors. For example, a DNA sequence for a signal peptide (secretory leader) may be operably linked to a CD40-L DNA

30 sequence. The signal peptide is expressed as a precursor amino acid sequence which enables improved extracellular secretion of translated fusion polypeptide by a yeast host cell.

Suitable host cells for expression of CD40-L polypeptides include prokaryotes, yeast or higher eukaryotic cells. Prokaryotes include gram negative or gram positive

35 organisms, for example, *E. coli* or *Bacilli.* Suitable prokaryotic host cells for transformation include, for example, *E. coli, Bacillus subtilis, Salmonella typhimurium,* and various other species within the genera *Pseudomonas,*

Streptomyces, and *Staphylococcus.* Higher eukaryotic cells include established cell lines of mammalian origin. Cell-free translation systems could also be employed to produce CD40-L polypeptides using RNAs derived from DNA constructs disclosed herein. Appropriate cloning and expression vectors for use with bacterial, fungal,

5   yeast, and mammalian cellular hosts are described, for example, in Pouwels et al. *Cloning Vectors: A Laboratory Manual,* Elsevier, New York, (1985).

In a prokaryotic host cell, such as *E. coli,* a CD40-L polypeptide or analog may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the

10   expressed recombinant CD40-L polypeptide. Prokaryotic host cells may be used for expression of CD40-L polypeptides that do not require extensive proteolytic or disulfide processing.

The expression vectors carrying the recombinant CD40-L DNA sequence are transfected or transformed into a substantially homogeneous culture of a suitable host

15   microorganism or mammalian cell line. Transformed host cells are cells which have been transformed or transfected with nucleotide sequences encoding CD40-L polypeptides and express CD40-L polypeptides. Expressed CD40-L polypeptides will be located within the host cell and/or secreted into culture supernatant fluid, depending upon the nature of the host cell and the gene construct inserted into the host cell.

20   Expression vectors transfected into prokaryotic host cells generally comprise one or more phenotypic selectable markers. A phenotypic selectable marker is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement, and an origin of replication recognized by the host to ensure amplification within the host. Other useful expression vectors for prokaryotic host cells

25   include a selectable marker of bacterial origin derived from commercially available plasmids. This selectable marker can comprise genetic elements of the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. The pBR322 "backbone" sections are combined with an appropriate promoter and a CD40-

30   L DNA sequence. Other commercially vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA).

Promoter sequences are commonly used for recombinant prokaryotic host cell expression vectors. Common promoter sequences include β-lactamase (penicillinase),

35   lactose promoter system (Chang et al., *Nature 275:*615, 1978; and Goeddel et al., *Nature 281:*544, 1979), tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res. 8:*4057, 1980; and EP-A-36776) and tac promoter (Maniatis, *Molecular Cloning:*

A Laboratory Manual, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful prokaryotic host cell expression system employs a phage λ P$_L$ promoter and a cI857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ P$_L$ promoter include plasmid pHUB2 (resident in *E. coli* strain JMB9 (ATCC 37092)) and pPLc28 (resident in *E. coli* RR1 (ATCC 53082)).

CD40-L may be expressed in yeast host cells, preferably from the *Saccharomyces* genus (e.g., *S. cerevisiae*). Other genera of yeast, such as *Pichia* or *Kluyveromyces*, may also be employed. Yeast vectors will often contain an origin of replication sequence from a 2μ yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, and sequences for transcription termination. Preferably, yeast vectors include an origin of replication sequence and selectable marker. Suitable promoter sequences for yeast vectors include promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7:149, 1968; and Holland et al., *Biochem.* 17:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EPA-73,657.

Yeast vectors can be assembled, for example, using DNA sequences from pBR322 for selection and replication in *E. coli* (Amp^r gene and origin of replication). Other yeast DNA sequences that can be included in a yeast expression construct include a glucose-repressible ADH2 promoter and α-factor secretion leader. The ADH2 promoter has been described by Russell et al. (*J. Biol. Chem.* 258:2674, 1982) and Beier et al. (*Nature* 300:724, 1982). The yeast α-factor leader sequence directs secretion of heterologous polypeptides. The α-factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. *See, e.g.,* Kurjan et al., *Cell* 30:933, 1982 and Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, 1984. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929, 1978. The Hinnen et al. protocol selects for Trp$^+$ transformants in a selective medium,

23

wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 µg/ml adenine and 20 µg/ml uracil.

Yeast host cells transformed by vectors containing ADH2 promoter sequence may be grown for inducing expression in a "rich" medium. An example of a rich

5   medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 µg/ml adenine and 80 µg/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

Mammalian or insect host cell culture systems could also be employed to express recombinant CD40-L polypeptides. Examples of suitable mammalian host cell

10   lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651) (Gluzman et al., *Cell 23*:175, 1981), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, and BHK (ATCC CRL 10) cell lines. Suitable mammalian expression vectors include nontranscribed elements such as an origin of replication, a promoter sequence, an enhancer linked to the structural gene, other 5' or

15   3' flanking nontranscribed sequences, such as ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences.

Transcriptional and translational control sequences for mammalian host cell expression vectors may be excised from viral genomes. For example, commonly used

20   mammalian cell promoter sequences and enhancer sequences are derived from Polyoma virus, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide the other genetic elements required for expression of a structural gene sequence in a

25   mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication (Fiers et al., *Nature 273*:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* I site located in the SV40 viral origin of

30   replication site is included.

Exemplary mammalian expression vectors can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol. 3*:280, 1983). A useful high expression vector, PMLSV N1/N4, described by Cosman et al., *Nature 312*:768, 1984 has been deposited as ATCC 39890. Additional useful mammalian expression vectors are described in EP-

35   A-0367566, and in U.S. Patent Application Serial No. 07/701,415, filed May 16, 1991, incorporated by reference herein. For expression of a type II protein extracellular region, such as CD40-L, a heterologous signal sequence should be added,

such as the signal sequence for interleukin-7 (IL-7) described in United States Patent 4,965,195, or the signal sequence for interleukin-2 receptor described in United States Patent Application 06/626,667 filed on July 2, 1984.

Human or murine CD40-L can be made in membrane-bound form when an
5 intracellular and transmembrane regions are included or in soluble form with only the extracellular domain. We expressed full length murine CD40-L in mammalian cells to yield cells expressing membrane-bound murine CD40-L. CV1 cells were transfected with a cDNA shown in Figure 1 (SEQ ID NO:1) in HAVEO vector or CV1 cells were transfected with HAVEO empty vector using techniques described in Example 6 herein.
10 This yielded transfected CV1 cells expressing membrane-bound murine CD40-L. These cells were used as a source of membrane-bound murine CD40-L for the series of experiments reported in Examples 10 -13 reported below.

Purification of Recombinant CD40-L Polypeptides

CD40-L polypeptides may be prepared by culturing transformed host cells
15 under culture conditions necessary to express CD40-L polypeptides. The resulting expressed polypeptides may then be purified from culture media or cell extracts. A CD40-L polypeptide, if desired, may be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification
20 matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices
25 comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred.

Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, (e.g., silica gel having pendant methyl or other aliphatic groups) can be employed to further purify CD40-L. Some or all of the foregoing purification steps, in various combinations, can also be
30 employed to provide a substantially homogeneous recombinant protein.

It is also possible to utilize an affinity column comprising CD40 ligand binding domain to affinity-purify expressed CD40-L polypeptides. CD40-L polypeptides can be removed from an affinity column in a high salt elution buffer and then dialyzed into a lower salt buffer for use.

35 Recombinant protein produced in bacterial culture is usually isolated by initial disruption of the host cells, centrifugation, extraction from cell pellets if an insoluble polypeptide, or from the supernatant fluid if a soluble polypeptide, followed by one or

more concentration, salting-out, ion exchange, affinity purification or size exclusion
chromatography steps. Finally, RP-HPLC can be employed for final purification
steps. Microbial cells can be disrupted by any convenient method, including freeze-
thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

5   Transformed yeast host cells are preferably employed to express CD40-L as a
secreted polypeptide. This simplifies purification. Secreted recombinant polypeptide
from a yeast host cell fermentation can be purified by methods analogous to those
disclosed by Urdal et al. (*J. Chromatog*. *296*:171, 1984). Urdal et al. describe two
sequential, reversed-phase HPLC steps for purification of recombinant human IL-2 on
10   a preparative HPLC column.

          Administration of CD40-L Compositions

          The present invention provides therapeutic compositions comprising an
effective amount of CD40-L in a suitable diluent or carrier and methods of treating
mammals using the compositions. For therapeutic use, purified CD40-L or a
15   biologically active analog thereof is administered to a patient, preferably a human, for
treatment in a manner appropriate to the indication. Thus, for example, CD40-L
pharmaceutical compositions (for example, in the form of a soluble extracellular
domain, or a fragment thereof) which is administered to achieve a desired therapeutic
effect can be given by bolus injection, continuous infusion, sustained release from
20   implants, or other suitable technique. Typically, a CD40-L therapeutic agent will be
administered in the form of a pharmaceutical composition comprising purified CD40-L
polypeptide in conjunction with physiologically acceptable carriers, excipients or
diluents. Such carriers will be nontoxic to patients at the dosages and concentrations
employed. Ordinarily, the preparation of such compositions entails combining a CD40-
25   L polypeptide with buffers, antioxidants such as ascorbic acid, low molecular weight
(less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates
including glucose, sucrose or dextrans, chelating agents such as EDTA, glutathione and
other stabilizers and excipients. Neutral buffered saline or saline mixed with
conspecific serum albumin are exemplary appropriate diluents. CD40-L sense or
30   antisense oligonucleotides may be administered *in vivo* by administering an effective
amount of a vector containing a nucleic acid sequence that encodes and effective
antisense or sense oligonucleotide. Additionally, CD40-L sense or antisense
oligonucleotides may be administered *ex vivo* by removing cells containing CD40-L
DNA or mRNA from an individual, incorporating an antisense or sense oligonucleotide
35   into the cells using gene transfer techniques, and re-infusing the cells into the
individual.

The following examples are intended to illustrate particular embodiments and not limit the scope of the invention.

EXAMPLE 1

5   This example describes construction of a CD40/Fc DNA construct to express a soluble CD40/Fc fusion protein for use in detecting cDNA clones encoding a CD40 ligand. The cDNA sequence of the extracellular region or ligand binding domain of complete CD40 human receptor sequence was obtained using polymerase chain reaction (PCR) techniques, and is based upon the sequence published in Stamenkovic et al.,

10   *supra*. A CD40 plasmid (CDM8) was used as a template for PCR amplification. CDM8 is described in Stamenkovic et al. and was obtained from the authors. A PCR technique (Sarki et al., *Science* 239:487, 1988) was employed using 5' (upstream) and 3' (downstream) oligonucleotide primers to amplify the DNA sequences encoding CD40 extracellular ligand binding domain. Upstream oligonucleotide primer 5'-

15   CCGTCGACCACCATGGTTCGTCTGCC -3' (SEQ ID NO:5) introduces a *Sal* 1 site upstream from an initiator methionine of CD40 and a downstream oligonucleotide primer 5'-ACAAGATCTGGGCTCTACGTATCTCAGCCGATCCTGGGGAC-3' (SEQ ID NO:7) that inserts amino acids Tyr Val Glu Pro Arg (SEQ ID NO:8) after amino acid 193 of CD40. Glu and Pro are the first two amino acids of a hinge region

20   of human IgG1, and are followed by a *Bgl* II restriction site that was used to fuse the extracellular domain of CD40 to the remained of human IgG1 Fc region.

The DNA construct pDC406/CD40/Fc was transfected into the monkey kidney cell line CV-1/EBNA (ATCC CRL 10478). The pDC406 plasmid includes regulatory sequences derived from SV40, human immunodeficiency virus (HIV), and Epstein-

25   Barr virus (EBV). The CV-1/EBNA cell line was derived by transfection of the CV-1 cell line with a gene encoding Epstein-Barr virus nuclear antigen-1 (EBNA-1) that constitutively expresses EBNA-1 driven from the human CMV intermediate-early enhancer/promoter. The EBNA-1 gene allows for episomal replication of expression vectors, such as pDC406, that contain the EBV origin of replication.

30   Once cells expressing the fusion construct were identified, large scale cultures of transfected cells were grown to accumulate supernatant from cells expressing CD40/Fc. The CD40/Fc fusion protein in supernatant fluid was purified by affinity purification. Briefly, one liter of culture supernatant containing the CD40/Fc fusion protein was purified by filtering mammalian cell supernatants (e.g., in a 0.45μ filter)

35   and applying filtrate to a protein A/G antibody affinity column (Schleicher and Schuell, Keene, NH) at 4°C at a flow rate of 80 ml/hr for a 1.5 cm x 12.0 cm column. The column was washed with 0.5 M NaCl in PBS (phosphate buffered saline) until free

protein could not be detected in wash buffer. Finally, the column was washed with PBS. Bound fusion protein was eluted from the column with 25 mM citrate buffer, pH 2.8, and brought to pH 7 with 500 mM Hepes buffer, pH 9.1. Silver-stained SDS gels of the eluted CD40/Fc fusion protein showed it to be > 98% pure.

5   Purified CD40/Fc fusion protein was iodinated with ^{125}I using a commercially available solid phase agent (IODO-GEN, Pierce). In this procedure, 5 μg of IODO-GEN were plated at the bottom of a 10 x 75 mm glass tube and incubated for twenty minutes at 4° C with 75 μl of 0.1 M sodium phosphate, pH 7.4 and 20 μl (2 mCi) Na^{125}I. The solution was then transferred to a second glass tube containing 5 μg of

10   CD40/Fc in 45 μl PBS and this reaction mixture was incubated for twenty minutes at 4° C. The reaction mixture was fractionated by gel filtration on a 2 ml bed volume of Sephadex® G-25 (Sigma), and then equilibrated in RPMI 1640 medium containing 2.5% (v/v) bovine serum albumin (BSA), 0.2% (v/v) sodium azide and 20 mM Hepes, pH 7.4 binding medium. The final pool of ^{125}I CD40/Fc was diluted to a working

15   stock solution of 1×10^{-7} M in binding medium and stored for up to one month at 4° C without detectable loss of receptor binding activity.

       Approximately 50% - 60% label incorporation was observed. Radioiodination yielded specific activities in the range of 1×10^{15} to 5×10^{15} cpm/nmole (0.42 - 2.0 atoms of radioactive iodine per molecule of protein). SDS polyacrylamide gel

20   electrophoresis (SDS-PAGE) revealed a single labeled polypeptide consistent with expected values. The labeled fusion protein was greater than 98% trichloroacetic acid (TCA) precipitable, indicating that the ^{125}I was covalently bound to the protein.

EXAMPLE 2

25   This example describes selection of a cell line putatively expressing CD40-L. Several cell lines were screened using the radioiodinated CD40/Fc fusion protein described in Example 1. Briefly, quantitative binding studies were performed according to standard methodology, and Scatchard plots were derived for the various cell lines. A clonal cell line (EL4, ATCC Catalog TIP 39) a murine thymoma cell line

30   was identified and sorted. Prior to sorting, EL-4 cells were found to express approximately 450 molecules of CD40-L per cell. The seventh sort cells were called EL-40.7 and were grown and found to express approximately 10,000 molecules of CD40-L per cell. Lastly, the ninth sort cells were called EL-40.9 and were grown and found to express approximately 15,000 molecules of CD40-L per cell.

EXAMPLE 3

This example describes preparation of a cDNA library for expression cloning of murine CD40-L. The library was prepared from a fifth sorted clone of a mouse thymoma cell line EL-4 (ATCC TIB 39), called EL-40.5. EL-40.5 cells were EL4 cells sorted five times with biotinylated CD40/Fc fusion protein in a FACS (fluorescence activated cell sorter). A cDNA library was made from RNA obtained from EL-40.5 cells essentially as described in US Patent 4,968,607, the disclosure of which is incorporated by reference herein. Briefly, a cDNA library was constructed by reverse transcription of poly (A)$^+$ mRNA isolated from the total RNA extracted from the EL-40.5 cell line. The library construction technique was substantially similar to that described by Ausubel et al., eds., *Current Protocols In Molecular Biology*, Vol. 1, (1987). Poly (A)$^+$ mRNA was isolated by oligo dT cellulose chromatography and double-stranded cDNA was made substantially as described by Gubler et al., *Gene* 25:263, 1983. Poly(A)$^+$ mRNA fragments were converted to RNA-cDNA hybrids by reverse transcriptase using random hexanucleotides as primers. The RNA-cDNA hybrids were then converted into double-stranded cDNA fragments using RNAase H in combination with DNA polymerase I. The resulting double-stranded cDNA was blunt-ended with T4 DNA polymerase.

Sal I adaptors

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5'- TCG ACT GGA ACG AGA CGA CCT GCT -3'
       GA CCT TGC TCT GCT GGA CGA - 5'
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were ligated to 5' ends of resulting blunt-ended cDNA, as described in Haymerle et al., *Nucleic Acids Res.* 14:8615, 1986. Non-ligated adaptors were removed by gel filtration chromatography at 68°C. This left 24 nucleotide non-self-complementary overhangs on cDNA. The same procedure was used to convert 5' *Sal* I ends of the mammalian expression vector pDC406 to 24 nucleotide overhangs complementary to those added to cDNA. Optimal proportions of adaptored vector and cDNA were ligated in the presence of T4 polynucleotide kinase. Dialyzed ligation mixtures were electroporated into *E. coli* strain DH5α and transformants selected on ampicillin plates.

Plasmid DNA was isolated from pools consisting of approximately 2,000 clones of transformed *E. coli* per pool. The isolated DNA was transfected into a sub-confluent layer of CV1-EBNA cells using DEAE-dextran followed by chloroquine treatment substantially according to the procedures described in Luthman et al., *Nucl. Acids Res.* 11:1295, 1983 and McCutchan et al., *J. Natl. Cancer Inst.* 41:351, 1986.

CV1-EBNA cells were maintained in complete medium (Dulbecco's modified Eagles' media containing 10% (v/v fetal calf serum, 50 U/ml penicillin, 50 U/ml streptomycin, and 2 mM L-glutamine) and were plated to a density of approximately 2

x 10^5 cells/well in single-well chambered slides (Lab-Tek). The slides were pre-treated with 1 ml human fibronectin (10 μg/ml PBS) for 30 minutes followed by a single washing with PBS. Media was removed from adherent cells growing in a layer and replaced with 1.5 ml complete medium containing 66.6μM chloroquine sulfate. About

5   0.2 ml of a DNA solution (2 μg DNA, 0.5 mg/ml DEAE-dextran in complete medium containing chloroquine) was added to the cells and the mixture was incubated at 37°C for about five hours. Following incubation, media was removed and the cells were shocked by addition of complete medium containing 10% DMSO (dimethylsulfoxide) for 2.5 - 20 minutes. Shocking was followed by replacement of the solution with fresh

10   complete medium. The cells were grown in culture for two to three days to permit transient expression of the inserted DNA sequences. These conditions led to a 30% to 80% transfection frequency in surviving CV1-EBNA cells.

EXAMPLE 4

15   This example describes screening of the expression cloning library made in Example 3 with a labeled CD40/Fc fusion protein made in Example 1. After 48 - 72 hours, transfected monolayers of CV1-EBNA cells made in Example 3 were assayed by slide autoradiography for expression of CD40-L using radioiodinated CD40/Fc fusion protein as prepared in Example 1. Transfected CV1-EBNA cells were washed

20   once with binding medium (RPMI 1640 containing 25 mg/ml bovine serum albumin (BSA), 2 mg/ml sodium azide, 20 mM Hepes pH 7.2, and 50 mg/ml nonfat dry milk) and incubated for 2 hours at 4°C ml in binding medium containing 1 x 10^{-9} M ^{125}I-CD40/Fc fusion protein. After incubation, cells in the chambered slides were washed three times with binding buffer, followed by two washes with PBS, (pH 7.3) to

25   remove unbound radiolabeled fusion protein.

          The cells were fixed by incubating in 10% gluteraldehyde in PBS (30 minutes at room temperature), washed twice in PBS and air-dried. The slides were dipped in Kodak GTNB-2 photographic emulsion (6x dilution in water) and exposed in the dark for two to four days days at room temperature in a light-proof box. The slides were

30   developed in Kodak D19 developer, rinsed in water and fixed in Agfa G433C fixer. The slides were individually examined under a microscope at 25-40x magnification. Positive slides showing cells expressing CD40-L were identified by the presence of autoradiographic silver grains against a light background.

          One pool containing approximately 2000 individual clones was identified as potentially positive for binding the CD40/Fc fusion protein. The pool was titered and

35   plated to provide plates containing approximately 200 colonies each. Each plate was scraped to provide pooled plasmid DNA for transfection into CV1-EBNA cells

according to the same procedure described above. The smaller pools were screened by slide autoradiography as described previously. One of the smaller pools contained clones that were positive for CD40-L as indicated by the presence of an expressed gene product capable of binding to the CD40/Fc fusion protein.

The positive smaller pool was titered and plated to obtain individual colonies. Approximately 400 individual colonies were picked and inoculated into culture medium in individual wells of 96-well plates. Cultures were mixed by pooling rows and columns and the mixed cultures were used to prepare DNA for a final round of transfection and screening. An intersection of a positive row and and a positive column indicated a potential positive colony. Ten potential positive colonies (i.e., candidate clones) were identified. DNA was isolated from each candidate clone, retransfected and rescreened. Five candidate clones were positive by binding to CD40/Fc. All five positive candidate clones contained a cDNA insert of 1468 nucleotides, as determined by dideoxynucleotide sequencing. The cDNA coding region of the CD40-L clone corresponds to the sequence of Figure 1 and SEQ ID NO:1.

A cloning vector containing murine CD40-L sequence, designated pDC406-mCD40-L, was deposited with the American Type Culture Collection, Rockville, MD (ATCC) on December 6, 1991, under accession number 68872. The nucleotide sequence and predicted amino acid sequence of this clone are illustrated in SEQ ID NO:1 and in Figure 1.

EXAMPLE 5

This example illustrates a cross-species hybridization technique which was used to isolate a human CD40-L homolog using a probe designed from the sequence of murine CD40-L. A murine CD40-L probe was produced by excising the coding region from murine CD40-L clone pDC406-CD40-L (nucleotide 13 through 793) and ^{32}P-labeling the fragment using random primers (Boehringer-Mannheim).

A human peripheral blood lymphocyte (PBL) cDNA library was constructed in a λ phage vector using λgt10 arms and packaged *in vitro* using a commercially available kit (Gigapak® Stratagene, San Diego, CA) according to the manufacturer's instructions. The PBL cells were obtained from normal human volunteers and treated with 10 ng/ml of OKT3 (an anti-CD3 antibody), and 10 ng/ml of human IL-2 (Immunex, Seattle, WA) for six days. The PBL cells were washed and stimulated with 500 ng/ml ionomycin (Calbiochem) and 10 ng/ml PMA (Sigma) for four hours. Messenger RNA and cDNA were obtained from the stimulated PBL cells and packaged into λgt10 phage vectors (Gigapak® Stratagene) according to manufacturer's instructions.

The murine probe was hybridized to phage cDNA in 6 X SSC (15 mM trisodium citrate, and 165 mM sodium chloride), 1 X Denhardt's solution, 2 mM EDTA, 0.5% Np40 at 63°C overnight. Hybridization was followed by extensive washing in 3 X SSC, 0.1% SDS at approximately 55°C for three hours. Specific

5   bands were visualized by autoradiography.

A cloning vector containing human CD40-L sequence, designated hCD40-L, was deposited with the American Type Culture Collection, Rockville, MD (ATCC) on December 6, 1991, under accession number 68873. The nucleotide sequence and predicted amino acid sequence of this clone are illustrated SEQ ID NO:11 and in Figure

10   2.

EXAMPLE 6

This example illustrates the expression of membrane-bound murine CD40-L in CV1-EBNA cells. Murine CD40-L cDNA in HAVEO vector or empty HAVEO vector

15   were transfected into CV1 EBNA cells using standard techniques, such as those described in McMahan et al. et al. *EMBO J.* 10:2821, 1991 and in Example 3 herein. Briefly, CV1 EBNA cells were plated at a density of 2×10^6 cells per 10 cm dish in 10 ml of Dulbecco's Minimal Essential Medium supplemented with 10% fetal calf serum (Medium). The cells were allowed to adhere overnight at 37°C. The Medium was

20   replaced with 1.5 ml of Medium containing 66.7 μM chloroquine and a DNA mixture containing 5 μg of cDNA encoding mCD40-L. Medium containing 175 μl, and 25 μl of DEAE dextran (4 mg/ml in PBS) was also added to the cells. The cells and cDNA were incubated at 37°C for 5 hours. The cDNA mixture was removed and the cells were shocked with 1 ml of fresh Medium containing 10% DMSO for 2.5 min. The

25   Medium was replaced with fresh Medium and the cells were grown for at least 3 days.

EXAMPLE 7

This example illustrates the preparation of monoclonal antibodies to CD40-L. Preparations of purified murine CD40-L or human CD40-L are prepared by COS cell

30   expression and CD40/Fc affinity purification as described herein. Purified CD40-L can generate monoclonal antibodies against CD40-L using conventional techniques, for example, those techniques described in U.S. Patent 4,411,993. Briefly, mice are immunized with CD40-L as an immunogen emulsified in complete Freund's adjuvant, and injected in amounts ranging from 10-100 μg subcutaneously or intraperitoneally.

35   Ten to twelve days later, the immunized animals are boosted with additional CD40-L emulsified in incomplete Freund's adjuvant. Mice are periodically boosted thereafter on a weekly to bi-weekly immunization schedule. Serum samples are periodically taken

by retro-orbital bleeding or tail-tip excision for testing by dot blot assay or ELISA (Enzyme-Linked Immunosorbent Assay), for CD40-L antibodies.

Following detection of an appropriate antibody titer, positive animals are provided one last intravenous injection of CD40-L in saline. Three to four days later, the animals are sacrificed, spleen cells harvested, and spleen cells are fused to a murine myeloma cell line (e.g., NS1 or Ag 8.653). Fusions generate hybridoma cells, which are plated in multiple microtiter plates in a HAT (hypoxanthine, aminopterin and thymidine) selective medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells are screened by ELISA for reactivity against purified CD40-L by adaptations of the techniques disclosed in Engvall et al., *Immunochem.* 8:871, 1971 and in U.S. Patent 4,703,004. Positive hybridoma cells can be injected intraperitoneally into syngeneic BALB/c mice to produce ascites containing high concentrations of anti-CD40-L monoclonal antibodies. Alternatively, hybridoma cells can be grown *in vitro* in flasks or roller bottles by various techniques. Monoclonal antibodies produced in mouse ascites can be purified by ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can also be used, as can affinity chromatography based upon binding to CD40-L.

EXAMPLE 8

This example illustrates anti-allergy therapeutic effects of sCD40 and CD40/Fc fusion protein. Soluble CD40 and CD40/Fc were tested for their ability to inhibit IL-4 (5 ng/ml) induced IgE secretion in a two donor MLC system The data from three experiments are presented in Table 1.

Table 1

	IgE (ng/ml)		
Addition	Exp. 1	Exp. 2	Exp. 3
medium	<0.1	<0.1	<0.1
IL-4	24	47	54
IL-4 + sCD40 (0.1 µg/ml)	19	nd	38
IL-4 + sCD40 (0.3 µg/ml)	14	29	24
IL-4 + sCD40 (1 µg/ml)	10	24	8
IL-4 + sCD40 (3 µg/ml)	7	19	2
IL-4 + IL-7R/Fc (10 µg/ml)	21	nd	58

IgE levels were measured after 12 days in culture by an ELISA procedure. Briefly, flat-bottomed 96-well microtiter plates (Corning) were coated with mouse mAb anti-human IgE (Zymed) at 1:500 dilution in PBS (phosphate buffered saline). After washing 3X, a blocking step was performed using 5% non-fat dried milk, followed by titration of human IgE standards or test supernatants. After washing 3X, biotinylated goat anti-human IgE (Kirkegaard and Perry) was added at a 1:500 dilution. This was followed by further washing and then addition of streptavidin-HRP (Zymed) at a 1:500 dilution. After further washing, the reaction was developed using TMB substrate (Kirkegaard and Perry) and absorbance measured at 520 nm. All washing steps were carried out in PBS plus 0.05% Tween. All incubation steps were performed at volumes of 100 μl/well for one hour at room temperature. The sensitivity of this assay is 100 pg/ml.

EXAMPLE 9

This example illustrates the effects of sCD40 and CD40/Fc fusion protein to inhibit soluble CD23 shedding from IL-4 (5 ng/ml) stimulated B cells. Soluble CD40 and CD40/Fc were tested for their ability to inhibit IL-4-induced sCD23 shedding in a two donor MLC system The data from three experiments are presented in Table 2.

Table 2

	sCD23 (ng/ml)					
Addition	Exp. 1		Exp. 2		Exp. 3	
	day 6	day 12	day 6	day 12	day 6	day 12
E⁻ + medium	55	<0.5	24	10	10	5
+ IL-4	115	55	96	62	44	27
+ IL-4 + sCD40 (1 μg/ml)	nd	nd	88	36	38	9
+ IL-4 + sCD40 (3 μg/ml)	97	4	82	31	40	4
+ IL-4 + sCD40 (10 μg/ml)	nd	nd	72	28	nd	nd
+ IL-4 + IL-7R/Fc (3 μg/ml)	111	48	103	67	40	22
PBM + medium	12	<0.5	15	5	3	10
+ IL-4	39	255	47	22	48	26
+ IL-4 + sCD40 (1 μg/ml)	nd	nd	44	18	46	18
+ IL-4 + sCD40 (3 μg/ml)	24	6	37	11	45	12
+ IL-4 + sCD40 (10 μg/ml)	nd	nd	28	5	nd	nd
+ IL-4 + IL-7R/Fc (3 μg/ml)	35	26	43	20	50	23

Soluble CD23 levels were measured after 6 and 12 days in culture by a commercial sCD23 ELISA detection kit (Binding Site, San Diego, CA). The sensitivity limit was 500 pg/ml. Approximately 1×10^5 cells per well were cultured in triplicate in round-bottomed 96-well microtiter plates (Intermountain Scientific, Bountiful UT) for
5 the indicated time in the presence or absence of additives as indicated in Table 2. The results show anti-allergy effects of sCD40. Similar studies were run with CD40/Fc (data not shown) instead of sCD40, and similar results were obtained. Accordingly, these data in Examples 8 and 9 illustrate an anti-allergy property for CD40.

EXAMPLE 10
10

This example illustrates B cell proliferative activity of membrane-bound murine CD40-L for human B cells. Human peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood from normal volunteers by density gradient centrifugation over Histopaque® (Sigma, St. Louis, MO) T cell-depleted preparations
15 of cells (E⁻) were obtained by removing T cells by rosetting with 2-aminoethylisothiouronium bromide-treated SRBC (sheep red blood cells) and further density gradient centrifugation over Histopaque®. B cell proliferation assays were conducted with E⁻ preparations in RPMI media with added 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a 10% CO_2 atmosphere. Approximately 1×10^5 E⁻
20 cells per well were cultured in triplicate in flat-bottomed 96-well microtiter plates (Corning) for 7 days in the presence of transfected CV1 EBNA cells (described in Example 6). The CV1 EBNA cells were transfected with murine CD40-L cDNA or empty vector. The cells were pulsed with 1 μCi/well of tritiated thymidine (25 Ci/nmole Amersham, Arlington Heights, IL) for the final eight hours of culture. Cells
25 were harvested onto glass fiber discs with an automated cell harvester and incorporated cpm were measured by liquid scintillation spectrometry.

Figure 4a shows a comparison of human B cell proliferation of CV1 EBNA cells transfected with empty vector (HAVEO) or with murine CD40-L cDNA in HAVEO vector. These data show that membrane-bound CD40-L stimulates human B
30 cell proliferation in the absence of a co-mitogen. Figure 4b shows a similar experiment, except that 10 ng/ml of human IL-4 was added to the cultures. In this experiment, IL-4 slightly enhances the B cell mitogenic activity of membrane-bound murine CD40-L. Figure 5 is a repeat of the experiment shown in Figure 4b. However, when the experiment was repeated, there was no evidence of IL-4 co-mitogenic
35 activity. There was repeated evidence of CD40-L mitogenic activity. Accordingly, membrane-bound CD40-L stimulates proliferation of human B cells.

EXAMPLE 11

This example illustrates the effect of membrane-bound murine CD40-L to stimulate IgE production and CD23 shedding from E⁻ cells isolated in Example 10. Approximately 1 X 10⁵ cells/well were cultured in triplicate round bottomed 96-well

5   Nunc microtiter plates (Intermountain Scientific, Bountiful UT) in Iscove's Modified Dulbecco's Medium (IMDM) plus 10% FCS in a humidified atmosphere of 10% CO_2. Medium was supplemented with 50 μg/ml human transferrin (Sigma), 0.5% bovine serum albumin (Sigma) and 1 μg/ml of each of oleic, linoleic and palmitic acids (Sigma). The E⁻ cells were cultured for 10 days in the presence of 5 ng/ml human IL-

10 4. A titration of CV1 EBNA cells transfected with murine CD40-L or empty vector were added. After ten days, culture supernatants were assayed for IgE by the ELISA procedure described in Example 8 or for CD23 shedding by the procedure described in Example 9.

Figure 6 shows a comparison of IgE production in the supernatants (in ng/ml)

15 for cultures of E⁻ cells and CV1 EBNA cells transfected with empty vector (HAVEO) or with CD40-L. No differences were noted with up to 3000 CV1 EBNA cells, however significant IgE production resulted with the addition of 10000 or 30000 CD40-L transfected CV1 EBNA cells. As a comparison, when E⁻ cells were incubated with medium alone, 5 ng/ml IL-4 or 5 ng/ml IL-4 plus 500 ng/ml G28-5 antibody, IgE

20 production was 4.7, 2.9 and >600 ng/ml, respectively. When CD23 shedding was measured in Figure 7, 10000 and 30000 CVI EBNA cells transfected with CD40-L showed increased CD23 shedding when compared to empty vector control CV1 EBNA cells. As a comparison, when E⁻ cells were incubated with medium alone, 5 ng/ml IL-4 or 5 ng/ml IL-4 plus 500 ng/ml G28-5 antibody, CD23 shedding was <0.1, 2.4 and

25 11.2 ng/ml, respectively. These data show that IgE production and CD23 shedding are both biological activities associated with membrane-bound CD40-L.

EXAMPLE 12

This example illustrates B cell proliferative activity, polyclonal immunoglobulin

30 (Ig) production, antigen-specific antibody formation and various method for using membrane-bound and soluble CD40-L in clinical applications. We obtained murine splenic B cells according to procedures described in Grabstein et al. I *supra*, Maliszewski et al. I *supra* and Maliszewski et al. II *supra*. Briefly, the mixed culture of cells was purified by T cell depletion using T cell antiserum and complement, and

35 adherent cell depletion by passage of Sephadex® G10 columns and by B cell positive selection by panning on petri dishes coated with goat anti-mouse IgM. Purified B cells were cultured in RPMI, fetal calf serum (5% for B cell proliferation assays and 20% for

plaque forming cell assays or polyclonal antibody assays), 2-mercaptoethanol, antibiotics, amino acids and pyruvate. B cell proliferation was measured according to the assay described in Example 10 and in Grabstein et al. I *supra*, Maliszewski et al. I *supra* and Maliszewski et al. II *supra*. Antigen-specific antibody formation was

5 measured by the procedure described in Grabstein et al., *J. Mol. Cell. Immunol.* 2:199, 1986 [Grabstein et al. II]. Briefly, antigen specific antibody formation used sheep red blood cells (SRBC) as antigen (0.03% v/v) in 2.0 ml cultures of 1×10^6 murine B cells per culture. The B cell cultures were incubated for 5 days and plaque forming cells were determined by Jerne hemolytic plaque assay as described in Grabstein et al.

10 II *supra*. Cell counts were determined in a coulter counter. Polyclonal Ig secretion was determined by isotype-specific ELISA assays in seven day cultures of 1×10^6 B cells per 2.0 ml culture as described in Maliszewski et al. I *supra* and Maliszewski et al. II *supra*.

The results of B cell proliferation by CV1 EBNA cells transfected with CD40-L

15 or empty vector or 7A1 cells (a T cell helper clone) are shown in Figures 8, 10 and 12. These data show that the greatest B cell proliferation was caused by CD40-L. T cell helper cells 7A1 and 7C2 had a minimal effect on B cell proliferation.

The effects of various cells upon antigen specific antibody formation are shown in Figures 9 and 11. Figure 9 shows a comparison of plaque forming cells comparing

20 T cell helper clone 7A1 and murine EL40.9 cells which secrete a soluble CD40-L. The EL40.9 cells seem to have an inhibitory effect upon antigen specific antibody formation. Figure 11 shows PFC (plaque forming cells) for T cell helper cells 7C2 and CV1 EBNA cells transfected with either empty vector or CD40-L. Both 7C2 cells and membrane-bound CD40-L stimulated antigen specific antibody formation (PFC).

25 Figure 13 compares antigen specific antibody formation of CD40-L and 7A1 cells in the presence or absence of 10 ng/ml interleukin-2 (IL-2). IL-2 increased PFC for 7A1 cells but did not increase PFC caused by membrane-bound CD40-L.

Polyclonal Ig production by murine B cells was compared for stimulation or inhibition with membrane-bound CD40-L, control CV1 EBNA cells and helper T cells

30 7A1 in the presence of cytokines IL-4 (10 ng/ml) and IL-5 (1:40 dilution of COS cell supernatants) or without added cytokines. The amount of IgA, IgG3, IgE, IgG2b, IgM and IgG1 are shown in Tables 3-8, respectively.

TABLE 3

IgA, ng/ml

	# CELLS	MEDIA	+IL-4+IL-5
CD40-L	2 X 10(5)	666.275 ± 174.444	64.639 ± 51.780
	1 X 10(5)	288.085 ± 20.773	291.831 ± 10.673
	1 X 10(4)	53.750 ± 36.531	910.072 ± 62.713
HAVEO	2 X 10(5)	0	628.190 ± 42.907
	1 X 10(5)	0	477.755 ± 57.478
	1 X 10(4)	0	295.640 ± 12.736
7A1 (2C11)	1 X 10(6)	0	2177.549 ± 377.052
	2 X 10(5)	0	646.898 ± 86.325
	1 X 10(5)	0	480.671 ± 40.011
MEDIA		0	458.152 ± 77.258
LPS		88.531 ± 31.248	132.336 ± 51.356

TABLE 4

IgG3, ng/ml

	# CELLS	MEDIA	+IL-4+IL-5
CD40-L	2 X 10(5)	108.427 ± 14.359	0
	1 X 10(5)	118.079 ± 8.021	46.535 ± 9.899
	1 X 10(4)	127.591 ± 6.268	467.023 ± 78.276
HAVEO	2 X 10(5)	0	29.773 ± 5.224
	1 X 10(5)	11.205 ± 4.434	66.323 ± 8.673
	1 X 10(4)	26.389 ± 10.221	34.671 ± 12.975
7A1 (2C11)	1 X 10(6)	33.420 ± 9.972	820.856 ± 39.442
	2 X 10(5)	0	436.074 ± 59.332
	1 X 10(5)	0	239.760 ± 45.978
MEDIA		21.808 ± 7.107	64.773 ± 13.924
LPS		816.697 ± 43.553	103.720 ± 11.883

TABLE 5

IgE, ng/ml

	# CELLS	MEDIA	+IL-4+IL-5
CD40-L	2 X 10(5)	0	64.144 ± 4.979
	1 X 10(5)	0	83.493 ± 9.093
	1 X 10(4)	0	461.155 ± 60.514
HAVEO	2 X 10(5)	0	0
	1 X 10(5)	0	4.208 ± .527
	1 X 10(4)	0	0
7A1 (2C11)	1 X 10(6)	0	208.091 ± 8.090
	2 X 10(5)	0	32.530 ± 0.723
	1 X 10(5)	0	15.889 ± 2.947
MEDIA		0	12.602 ± 1.460
LPS		0	408.355 ± 9.764

TABLE 6

IgG2b, ng/ml

	# CELLS	MEDIA	+IL-4+IL-5
CD40-L	2 X 10(5)	0	0
	1 X 10(5)	0	6.230 ± .285
	1 X 10(4)	0	47.414 ± .241
HAVEO	2 X 10(5)	0	7.001 ± 2.358
	1 X 10(5)	0	6.230 ± 2.285
	1 X 10(4)	0	9.620 ± 2.650
7A1 (2C11)	1 X 10(6)	0	189.343 ± 2.837
	2 X 10(5)	0	22.431 ± 6.835
	1 X 10(5)	0	7.207 ± 1.580
MEDIA		0	7.422 ± 1.602
LPS		0	33.291 ± 3.183

TABLE 7

IgM, µg/ml

	# CELLS	MEDIA	+IL-4+IL-5
CD40-L	2 X 10(5)	1.805 ± 0.639	0.439 ± 0.184
	1 X 10(5)	2.237 ± 0.583	5.878 ± 0.858
	1 X 10(4)	2.293 ± 0.595	96.730 ± 13.009
HAVEO	2 X 10(5)	0	10.890 ± 2.126
	1 X 10(5)	0	13.303 ± 0.993
	1 X 10(4)	0.624 ± 0.178	22.538 ± 2.304
7A1 (2C11)	1 X 10(6)	0.769 ± 0.124	104.857 ± 17.463
	2 X 10(5)	0.142 ± 0.052	27.016 ± 1.706
	1 X 10(5)	0.126 ± 0.048	13.070 ± 0.600
MEDIA		0.231 ± 0.057	36.809 ± 2.860
LPS		53.302 ± 9.668	41.974 ± 6.158

TABLE 8

IgG1, ng/ml

	# CELLS	MEDIA	+IL-4+IL-5
CD40-L	2 X 10(5)	0	130.185 ± 24.547
	1 X 10(5)	0	310.588 ± 1.261
	1 X 10(4)	0	270.727 ± 17.511
HAVEO	2 X 10(5)	0	187.668 ± 57.730
	1 X 10(5)	0	43.320 ± 49.770
	1 X 10(4)	0	1363.464 ± 45.841
7A1 (2C11)	1 X 10(6)	0	145.652 ± 136.070
	2 X 10(5)	0	365.563 ± 24.276
	1 X 10(5)	0	449.475 ± 101.012
MEDIA		0	133.660 ± 386.231
LPS		0	246.213 ± 21.526

These data indicate that the interaction of CD40 with its ligand is the principal molecular interaction responsible for T cell contact dependent induction of B cell growth and differentiation to both antigen-specific antibody production and polyclonal Ig secretion. As such, these data suggest that antagonists of this interaction, by soluble CD40, CD40/Fc fusion protein and possibly soluble CD40-L (monomeric), will

significantly interfere with development of antibody responses. Therefore clinical situations where CD40, CD40/Fc fusion proteins and soluble CD40-L include allergy, lupus, rheumatoid arthritis, insulin dependent diabetes mellitus, and any other diseases where autoimmune antibody or antigen/antibody complexes are responsible for clinical

5   pathology of the disease. Moreover, membrane-bound CD40-L or oligomeric soluble CD40-L will be useful to stimulate B cell proliferation and antibody production. As such, these forms of CD40-L are most useful for vaccine adjuvants and as a stimulating agent for mAb secretion from hybridoma cells.

EXAMPLE 13

10   This example illustrates the effect of membrane-bound CD40-L upon proliferation of and IgE secretion from peripheral blood mononuclear cells (E⁻). E- cells were obtained according to the procedure described in Example 10 and incubated for 7 or 10 days in the presence of CV1 EBNA cells transfected with empty vector or

15   mCD40-L cDNA. Additionally, CD40/Fc fusion protein (described in Example 1) or TNF Receptor/Fc fusion protein (described in WO 91/03553) was added to some of the preparations as indicated in Figure 14. IgE secretion was measured according to the procedure described in Example 8 and B cell proliferation was measured according to the procedure described in Example 10.

20   The results for B cell proliferation and IgE secretion are shown in Figure 14 for five different concentrations of transfected CV1 EBNA cells. Both B cell proliferation and IgE secretion were increased in the presence of membrane-bound CD40-L. Addition of CD40/Fc fusion protein ablated both B cell proliferation and IgE secretion. The TNF Receptor/Fc fusion protein had no effect. As a comparison for IgE secretion,

25   addition of IL-4 as a control agent (without transfected CV1 EBNA cells) produced no IgE in this assay and addition of IL-4 plus G28-5 anti-CD40 mAb resulted in 29.7 ng/ml IgE in this assay.

EXAMPLE 14

30   This example describes construction of a CD40-L/Fc DNA construct to express a soluble CD40-L/Fc fusion protein referred to as CD40-L/FC2 construct. DNA encoding CD40-L/FC2 comprises sequences encoding a leader (or signal) peptide, an eight amino acid hydrophilic sequence described by Hopp et al. (Hopp et al., *BioTechnology* 6:1204,1988; referred to as Flag®), a suitable Fc region of an

35   immunoglobulin, a [Gly₄Ser]₃ repeat sequence (described in U.S. Patent 5,073,627, which is incorporated by reference herein) or other suitable linker sequence, and the extracellular region of human CD40-L from amino acid 50 to amino acid 261 (SEQ ID

NO:11). A pDC406 expression vector containing a leader sequence, Flag®, and human IgG₁ Fc is prepared using conventional techniques of enzyme cutting and ligation of fragments encoding a leader sequence, Flag®, and human IgG₁ Fc, and restricted with *Nsi* 1 and *Not* 1.

5   A PCR technique (Sarki et al., *Science* 239:487, 1988) was employed using 5' (upstream) and 3' (downstream) oligonucleotide primers to amplify the DNA sequences encoding CD40 extracellular ligand binding domain from a cloning vector containing human CD40-L (ATCC 68873; SEQ ID NO: 11) to form a PCR fragment. The upstream oligonucleotide primer (SEQ ID NO:13) introduced a *Nsi* 1 site upstream

10   from a linker sequence ([Gly₄Ser]₃SerSer), which was followed by 21 nucleotides of the extracellular domain of CD40-L (amino acids 51 through 57 of SEQ ID NO: 11). A downstream oligonucleotide primer (SEQ ID NO:14) introduced a *Not* 1 site just downstream of the termination codon of the CD40-L. The PCR fragment was then ligated into the pDC406 expression vector containing a leader sequence, Flag®, and

15   human IgG₁ Fc. The nucleotide and predicted amino acid sequence of CD40-L/FC2 are presented in SEQ ID NO:15 and SEQ ID NO:16. The resultant DNA construct (CD40-L/FC2) was transfected into the monkey kidney cell line CV-1/EBNA (ATCC CRL 10478). The construct encoded a soluble CD40-L capable of binding CD40, as evidenced by binding observed in fluorescence-activated cell sorting (FACS) analysis

20   using cells that express CD40.

          Large scale cultures of human embryonic kidney 293 cells (ATCC CRL 1573) transfected with the construct encoding CD40-L/FC2 were grown to accumulate supernatant containing CD40-L/FC2. The 293 cell line, a permanent line of primary human embryonal kidney transformed by human adenovirus 5 DNA, permits

25   expression of recombinant proteins ligated into the pCD406 vector. The CD40-L/FC2 fusion protein in supernatant fluid was purified by affinity purification. Briefly, culture supernatant containing the CD40-L/FC2 fusion protein was purified by filtering mammalian cell supernatants (e.g., in a 0.45μ filter) and applying filtrate to an antibody affinity column comprising biotinylated goat anti-human IgG (Jackson Immunoresearch

30   Laboratories, Inc., Westgrove, PA, USA) coupled to Streptavidin-agarose (Pierce Chemical, Rockford, IL, USA) at 4°C, at a flow rate of approximately 60 to 80 ml/hr for a 1.5 cm x 12.0 cm column. The column was washed with approximately 20 column volumes of PBS (phosphate buffered saline), until free protein could not be detected in wash buffer. Bound fusion protein was eluted from the column with 12.5

35   mM citrate buffer, 75 mM NaCl, pH 2.8, and brought to pH 7 with 500 mM Hepes buffer, pH 9.1. The purified, oligomeric CD40-L/FC2 peptide induced human B cell proliferation in the absence of any co-stimuli, and (in conjunction with the appropriate

cytokine) resulted in the production of IgG, IgE, IgA and IgM, as described in Example 12 for membrane-bound CD40-L.

EXAMPLE 15

5   This example describes construction of a CD40-L DNA construct to express a soluble CD40-L fusion protein referred to as trimeric CD40-L. Trimeric CD40-L contains a leader sequence, a 33 amino acid sequence referred to as a "leucine zipper" (SEQ ID NO:17), and an eight amino acid hydrophilic sequence described by Hopp et al. (Hopp et al., *Bio/Technology* 6:1204,1988; referred to as Flag®), followed by the
10   extracellular region of human CD40-L from amino acid 50 to amino acid 261 (SEQ ID NO:11). The utility of the leader and the Flag® sequences have been described in the Detailed Description. The 33 amino acid sequence presented in SEQ ID NO:17 trimerizes spontaneously in solution. Fusion proteins comprising this 33 amino acid sequence are thus expected to form trimers or multimers spontaneously.

15   The construct is prepared by synthesizing oligonucleotides representing a leader sequence, the 33 amino acid sequence described above, and the Flag® sequence, then ligating the final product to a DNA fragment encoding amino acids 51 through 261 of SEQ ID NO:11, prepared as described in Example 14.

The resulting ligation product in expression vector pDC406 was transfected into
20   the monkey kidney cell line CV-1/EBNA (ATCC CRL 10478). The pDC406 plasmid includes regulatory sequences derived from SV40, human immunodeficiency virus (HIV), and Epstein-Barr virus (EBV). The CV-1/EBNA cell line was derived by transfection of the CV-1 cell line with a gene encoding Epstein-Barr virus nuclear antigen-1 (EBNA-1) that constitutively expresses EBNA-1 driven from the human
25   CMV intermediate-early enhancer/promoter. The EBNA-1 gene allows for episomal replication of expression vectors, such as pDC406, that contain the EBV origin of replication.

Once cells expressing the fusion construct are identified, large scale cultures of transfected cells are grown to accumulate supernatant from cells expressing trimeric
30   CD40-L. The trimeric CD40-L fusion protein in supernatant fluid is purified by affinity purification substantially as described in U.S. Patent 5,011,912. Silver-stained SDS gels of the eluted CD40-L fusion protein can be prepared to determine purity.

35   SEQUENCE LISTING

(1) GENERAL INFORMATION:

40   (i) APPLICANT: ARMITAGE, RICHARD

FANSLOW, WILLIAM
SPRIGGS, MELANIE

(ii) TITLE OF INVENTION: NOVEL CYTOKINE

5

(iii) NUMBER OF SEQUENCES: 17

(iv) CORRESPONDENCE ADDRESS:
    (A) ADDRESSEE: IMMUNEX CORPORATION
10   (B) STREET: 51 UNIVERSITY STREET
    (C) CITY: SEATTLE
    (D) STATE: WASHINGTON
    (E) COUNTRY: USA
    (F) ZIP: 98101

15

(v) COMPUTER READABLE FORM:
    (A) MEDIUM TYPE: Floppy disk
    (B) COMPUTER: IBM PC compatible
    (C) OPERATING SYSTEM: PC-DOS/MS-DOS
20   (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
    (A) APPLICATION NUMBER:
    (B) FILING DATE:
25   (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
    (A) NAME: OSTER, JEFFREY B.
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30   (C) REFERENCE/DOCKET NUMBER: 2802

(ix) TELECOMMUNICATION INFORMATION:
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    (B) TELEFAX: 2065870606

35

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
40   (A) LENGTH: 783 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO
50

(vi) ORIGINAL SOURCE:
    (A) ORGANISM: MOUSE

(vii) IMMEDIATE SOURCE:
55   (B) CLONE: CD40-L

(ix) FEATURE:
    (A) NAME/KEY: CDS
    (B) LOCATION: 1..783
60

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
        ATG ATA GAA ACA TAC AGC CAA CCT TCC CCC AGA TCC GTG GCA ACT GGA
        48
  5     Met Ile Glu Thr Tyr Ser Gln Pro Ser Pro Arg Ser Val Ala Thr Gly
        1               5                   10                  15

        CTT CCA GCG AGC ATG AAG ATT TTT ATG TAT TTA CTT ACT GTT TTC CTT
        96
 10     Leu Pro Ala Ser Met Lys Ile Phe Met Tyr Leu Leu Thr Val Phe Leu
                        20                  25                  30

        ATC ACC CAA ATG ATT GGA TCT GTG CTT TTT GCT GTG TAT CTT CAT AGA
        144
 15     Ile Thr Gln Met Ile Gly Ser Val Leu Phe Ala Val Tyr Leu His Arg
                    35                  40                  45

        AGA TTG GAT AAG GTC GAA GAG GAA GTA AAC CTT CAT GAA GAT TTT GTA
        192
 20     Arg Leu Asp Lys Val Glu Glu Glu Val Asn Leu His Glu Asp Phe Val
                50                  55                  60

        TTC ATA AAA AAG CTA AAG AGA TGC AAC AAA GGA GAA GGA TCT TTA TCC
        240
 25     Phe Ile Lys Lys Leu Lys Arg Cys Asn Lys Gly Glu Gly Ser Leu Ser
        65                  70                  75                  80

        TTG CTG AAC TGT GAG GAG ATG AGA AGG CAA TTT GAA GAC CTT GTC AAG
        288
 30     Leu Leu Asn Cys Glu Glu Met Arg Arg Gln Phe Glu Asp Leu Val Lys
                        85                  90                  95

        GAT ATA ACG TTA AAC AAA GAA GAG AAA AAA GAA AAC AGC TTT GAA ATG
        336
 35     Asp Ile Thr Leu Asn Lys Glu Glu Lys Lys Glu Asn Ser Phe Glu Met
                        100                 105                 110

        CAA AGA GGT GAT GAG GAT CCT CAA ATT GCA GCA CAC GTT GTA AGC GAA
        384
 40     Gln Arg Gly Asp Glu Asp Pro Gln Ile Ala Ala His Val Val Ser Glu
                    115                 120                 125

        GCC AAC AGT AAT GCA GCA TCC GTT CTA CAG TGG GCC AAG AAA GGA TAT
        432
 45     Ala Asn Ser Asn Ala Ala Ser Val Leu Gln Trp Ala Lys Lys Gly Tyr
                    130                 135                 140

        TAT ACC ATG AAA AGC AAC TTG GTA ATG CTT GAA AAT GGG AAA CAG CTG
        480
 50     Tyr Thr Met Lys Ser Asn Leu Val Met Leu Glu Asn Gly Lys Gln Leu
        145                 150                 155                 160

        ACG GTT AAA AGA GAA GGA CTC TAT TAT GTC TAC ACT CAA GTC ACC TTC
        528
 55     Thr Val Lys Arg Glu Gly Leu Tyr Tyr Val Tyr Thr Gln Val Thr Phe
                        165                 170                 175

        TGC TCT AAT CGG GAG CCT TCG AGT CAA CGC CCA TTC ATC GTC GGC CTC
        576
 60     Cys Ser Asn Arg Glu Pro Ser Ser Gln Arg Pro Phe Ile Val Gly Leu
                        180                 185                 190
```

TGG CTG AAG CCC AGC AGT GGA TCT GAG AGA ATC TTA CTC AAG GCG GCA
624
Trp Leu Lys Pro Ser Ser Gly Ser Glu Arg Ile Leu Leu Lys Ala Ala
        195   200   205

AAT ACC CAC AGT TCC TCC CAG CTT TGC GAG CAG CAG TCT GTT CAC TTG
672
Asn Thr His Ser Ser Ser Gln Leu Cys Glu Gln Gln Ser Val His Leu
        210   215   220

GGC GGA GTG TTT GAA TTA CAA GCT GGT GCT TCT GTG TTT GTC AAC GTG
720
Gly Gly Val Phe Glu Leu Gln Ala Gly Ala Ser Val Phe Val Asn Val
225   230   235   240

ACT GAA GCA AGC CAA GTG ATC CAC AGA GTT GGC TTC TCA TCT TTT GGC
768
Thr Glu Ala Ser Gln Val Ile His Arg Val Gly Phe Ser Ser Phe Gly
                245   250   255

TTA CTC AAA CTC TG
783
Leu Leu Lys Leu
                260

(2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 260 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ile Glu Thr Tyr Ser Gln Pro Ser Pro Arg Ser Val Ala Thr Gly
1   5   10   15

Leu Pro Ala Ser Met Lys Ile Phe Met Tyr Leu Leu Thr Val Phe Leu
                20   25   30

Ile Thr Gln Met Ile Gly Ser Val Leu Phe Ala Val Tyr Leu His Arg
                35   40   45

Arg Leu Asp Lys Val Glu Glu Glu Val Asn Leu His Glu Asp Phe Val
        50   55   60

Phe Ile Lys Lys Leu Lys Arg Cys Asn Lys Gly Glu Gly Ser Leu Ser
65   70   75   80

Leu Leu Asn Cys Glu Glu Met Arg Arg Gln Phe Glu Asp Leu Val Lys
                85   90   95

Asp Ile Thr Leu Asn Lys Glu Glu Lys Lys Glu Asn Ser Phe Glu Met
            100   105   110

Gln Arg Gly Asp Glu Asp Pro Gln Ile Ala Ala His Val Val Ser Glu
            115   120   125

46

```
        Ala Asn Ser Asn Ala Ala Ser Val Leu Gln Trp Ala Lys Lys Gly Tyr
            130                 135                 140

 5      Tyr Thr Met Lys Ser Asn Leu Val Met Leu Glu Asn Gly Lys Gln Leu
        145                 150                 155                 160

        Thr Val Lys Arg Glu Gly Leu Tyr Tyr Val Tyr Thr Gln Val Thr Phe
                        165                 170                 175

10      Cys Ser Asn Arg Glu Pro Ser Ser Gln Arg Pro Phe Ile Val Gly Leu
                        180                 185                 190

        Trp Leu Lys Pro Ser Ser Gly Ser Glu Arg Ile Leu Leu Lys Ala Ala
15              195                 200                 205

        Asn Thr His Ser Ser Ser Gln Leu Cys Glu Gln Gln Ser Val His Leu
            210                 215                 220

20      Gly Gly Val Phe Glu Leu Gln Ala Gly Ala Ser Val Phe Val Asn Val
        225                 230                 235                 240

        Thr Glu Ala Ser Gln Val Ile His Arg Val Gly Phe Ser Ser Phe Gly
                        245                 250                 255

25      Leu Leu Lys Leu
                    260
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30   (2) INFORMATION FOR SEQ ID NO:3:

            (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 740 base pairs
                (B) TYPE: nucleic acid
35   (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

40   (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

        (vi) ORIGINAL SOURCE:
45   (A) ORGANISM: HUMAN

        (vii) IMMEDIATE SOURCE:
                (B) CLONE: IgG1 Fc

50   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

        CGGTACCGCT AGCGTCGACA GGCCTAGGAT ATCGATACGT AGAGCCCAGA TCTTGTGACA
        60
55
        AAACTCACAC ATGCCCACCG TGCCCAGCAC CTGAACTCCT GGGGGGACCG TCAGTCTTCC
        120

        TCTTCCCCCC AAAACCCAAG GACACCCTCA TGATCTCCCG GACCCCTGAG GTCACATGCG
60   180

47

TGGTGGTGGA CGTGAGCCAC GAAGACCCTG AGGTCAAGTT CAACTGGTAC GTGGACGGCG
240

TGGAGGTGCA TAATGCCAAG ACAAAGCCGC GGGAGGAGCA GTACAACAGC ACGTACCGGG
300

TGGTCAGCGT CCTCACCGTC CTGCACCAGG ACTGGCTGAA TGGCAAGGAC TACAAGTGCA
360

AGGTCTCCAA CAAAGCCCTC CCAGCCCCCA TGCAGAAAAC CATCTCCAAA GCCAAAGGGC
420

AGCCCCGAGA ACCACAGGTG TACACCCTGC CCCCATCCCG GGATGAGCTG ACCAAGAACC
480

AGGTCAGCCT GACCTGCCTG GTCAAAGGCT TCTATCCCAG GCACATCGCC GTGGAGTGGG
540

AGAGCAATGG GCAGCCGGAG AACAACTACA AGACCACGCC TCCCGTGCTG GACTCCGACG
600

GCTCCTTCTT CCTCTACAGC AAGCTCACCG TGGACAAGAG CAGGTGGCAG CAGGGGAACG
660

TCTTCTCATG CTCCGTGATG CATGAGGCTC TGCACAACCA CTACACGCAG AAGAGCCTCT
720

CCCTGTCTCC GGGTAAATGA
740

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 519 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (A) ORGANISM: HUMAN

    (vii) IMMEDIATE SOURCE:
        (B) CLONE: CD40 EXTRACELLULAR REGION

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CAGAACCACC CACTGCATGC AGAGAAAAAC AGTACCTAAT AAACAGTCAG TGCTGTTCTT
60

TGTGCCAGCC AGGACAGAAA CTGGTGAGTG ACTGCACAGA GTTCACTGAA ACGGAATGCC
120

TTCCTTGCGG TGAAAGCGAA TTCCTAGACA CCTGGAACAG AGAGACACAC TGCCACCAGC
180

ACAAATACTG CGACCCCAAC CTAGGGCTTC GGGTCCAGCA GAAGGGCACC TCAGAAACAG
240

ACACCATCTG CACCTGTGAA GAAGGCTGGC ACTGTACGAG TGAGGCCTGT GAGAGCTGTG
300

TCCTGCACCG CTCATGCTCG CCCGGCTTTG GGGTCAAGCA GATTGCTACA GGGGTTTCTG
360

ATACCATCTG CGAGCCCTGC CCAGTCGGCT TCTTCTCCAA TGTGTCATCT GCTTTCGAAA
420

AATGTCACCC TTGGACAAGC TGTGAGACCA AAGACCTGGT TGTGCAACAG GCAGGCACAA
480

ACAAGACTGA TGTTGTCTGT GGTCCCCAGG ATCGGCTGA
519

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 26 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
       (A) ORGANISM: PCR PRIMER

    (vii) IMMEDIATE SOURCE:
       (B) CLONE: CD40 5' PRIMER

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCGTCGACCA CCATGGTTCG TCTGCC
26

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 28 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
       (A) ORGANISM: PCR PRIMER

    (vii) IMMEDIATE SOURCE:
            (B) CLONE: CD40 3' PRIMER

5
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

    CCGTCGACGT CTAGAGCCGA TCCTGGGG
    28
10
    (2) INFORMATION FOR SEQ ID NO:7:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 40 base pairs
15   (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA
20
    (iii) HYPOTHETICAL: NO

    (vi) ORIGINAL SOURCE:
            (A) ORGANISM: PCR PRIMER
25
    (vii) IMMEDIATE SOURCE:
            (B) CLONE: CD40 3' DOWNSTREAM PRIMER

30   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

    ACAAGATCTG GGCTCTACGT ACTCAGCCGA TCCTGGGGAC
    40
35   (2) INFORMATION FOR SEQ ID NO:8:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 5 amino acids
            (B) TYPE: amino acid
40   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (iii) HYPOTHETICAL: NO
45
    (v) FRAGMENT TYPE: internal

    (vi) ORIGINAL SOURCE:
            (A) ORGANISM: PENTAPEPTIDE
50
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

    Tyr Val Gly Pro Arg
55 1   5

    (2) INFORMATION FOR SEQ ID NO:9:

        (i) SEQUENCE CHARACTERISTICS:
60   (A) LENGTH: 43 base pairs
            (B) TYPE: nucleic acid

              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: cDNA

      (iii) HYPOTHETICAL: NO

      (vi) ORIGINAL SOURCE:
              (A) ORGANISM: PCR PRIMER

      (vii) IMMEDIATE SOURCE:
              (B) CLONE: HUMAN IGG1/FC 5' PRIMER

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TATTAATCAT TCAGTAGGGC CCAGATCTTG TGACAAAACT CAC
43

    (2) INFORMATION FOR SEQ ID NO:10:

        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 38 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: cDNA

      (iii) HYPOTHETICAL: NO

      (iv) ANTI-SENSE: NO

      (vi) ORIGINAL SOURCE:
              (A) ORGANISM: PCR PRIMER

      (vii) IMMEDIATE SOURCE:
              (B) CLONE: HUMAN IGG1/FC 3' DOWNSTREAM PRIMER

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCCAGCTTAA CTAGTTCATT TACCCGGAGA CAGGGAGA
38

    (2) INFORMATION FOR SEQ ID NO:11:

        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 840 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: cDNA

      (iii) HYPOTHETICAL: NO

      (iv) ANTI-SENSE: NO

      (vi) ORIGINAL SOURCE:
              (A) ORGANISM: Homo sapiens

51

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      (vii)  IMMEDIATE SOURCE:
               (B) CLONE: CD40-L

5       (ix) FEATURE:
               (A) NAME/KEY: CDS
               (B) LOCATION: 46..831


10      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

     TGCCACCTTC TCTGCCAGAA GATACCATTT CAACTTTAAC ACAGC ATG ATC GAA
     54
                                                          Met Ile Glu
15                                                          1


     ACA TAC AAC CAA ACT TCT CCC CGA TCT GCG GCC ACT GGA CTG CCC ATC
     102
     Thr Tyr Asn Gln Thr Ser Pro Arg Ser Ala Ala Thr Gly Leu Pro Ile
20        5               10                  15


     AGC ATG AAA ATT TTT ATG TAT TTA CTT ACT GTT TTT CTT ATC ACC CAG
     150
     Ser Met Lys Ile Phe Met Tyr Leu Leu Thr Val Phe Leu Ile Thr Gln
25    20              25              30                  35


     ATG ATT GGG TCA GCA CTT TTT GCT GTG TAT CTT CAT AGA AGG TTG GAC
     198
     Met Ile Gly Ser Ala Leu Phe Ala Val Tyr Leu His Arg Arg Leu Asp
30                    40              45                  50


     AAG ATA GAA GAT GAA AGG AAT CTT CAT GAA GAT TTT GTA TTC ATG AAA
     246
     Lys Ile Glu Asp Glu Arg Asn Leu His Glu Asp Phe Val Phe Met Lys
35                55                  60                  65


     ACG ATA CAG AGA TGC AAC ACA GGA GAA AGA TCC TTA TCC TTA CTG AAC
     294
     Thr Ile Gln Arg Cys Asn Thr Gly Glu Arg Ser Leu Ser Leu Leu Asn
40        70                  75                  80


     TGT GAG GAG ATT AAA AGC CAG TTT GAA GGC TTT GTG AAG GAT ATA ATG
     342
     Cys Glu Glu Ile Lys Ser Gln Phe Glu Gly Phe Val Lys Asp Ile Met
45        85                  90                  95


     TTA AAC AAA GAG GAG ACG AAG AAA GAA AAC AGC TTT GAA ATG CAA AAA
     390
     Leu Asn Lys Glu Glu Thr Lys Lys Glu Asn Ser Phe Glu Met Gln Lys
50   100                 105                 110                 115


     GGT GAT CAG AAT CCT CAA ATT GCG GCA CAT GTC ATA AGT GAG GCC AGC
     438
     Gly Asp Gln Asn Pro Gln Ile Ala Ala His Val Ile Ser Glu Ala Ser
55                    120                 125                 130


     AGT AAA ACA ACA TCT GTG TTA CAG TGG GCT GAA AAA GGA TAC TAC ACC
     486
     Ser Lys Thr Thr Ser Val Leu Gln Trp Ala Glu Lys Gly Tyr Tyr Thr
60                    135                 140                 145
```

```
ATG AGC AAC AAC TTG GTA ACC CTG GAA AAT GGG AAA CAG CTG ACC GTT
534
Met Ser Asn Asn Leu Val Thr Leu Glu Asn Gly Lys Gln Leu Thr Val
        150             155                 160

AAA AGA CAA GGA CTC TAT TAT ATC TAT GCC CAA GTC ACC TTC TGT TCC
582
Lys Arg Gln Gly Leu Tyr Tyr Ile Tyr Ala Gln Val Thr Phe Cys Ser
    165             170                 175

AAT CGG GAA GCT TCG AGT CAA GCT CCA TTT ATA GCC AGC CTC TGC CTA
630
Asn Arg Glu Ala Ser Ser Gln Ala Pro Phe Ile Ala Ser Leu Cys Leu
180             185             190                 195

AAG TCC CCC GGT AGA TTC GAG AGA ATC TTA CTC AGA GCT GCA AAT ACC
678
Lys Ser Pro Gly Arg Phe Glu Arg Ile Leu Leu Arg Ala Ala Asn Thr
                200             205             210

CAC AGT TCC GCC AAA CCT TGC GGG CAA CAA TCC ATT CAC TTG GGA GGA
726
His Ser Ser Ala Lys Pro Cys Gly Gln Gln Ser Ile His Leu Gly Gly
                215             220             225

GTA TTT GAA TTG CAA CCA GGT GCT TCG GTG TTT GTC AAT GTG ACT GAT
774
Val Phe Glu Leu Gln Pro Gly Ala Ser Val Phe Val Asn Val Thr Asp
            230             235             240

CCA AGC CAA GTG AGC CAT GGC ACT GGC TTC ACG TCC TTT GGC TTA CTC
822
Pro Ser Gln Val Ser His Gly Thr Gly Phe Thr Ser Phe Gly Leu Leu
    245             250             255

AAA CTC TGAACAGTGT CA
840
Lys Leu
260
```

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 261 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Met Ile Glu Thr Tyr Asn Gln Thr Ser Pro Arg Ser Ala Ala Thr Gly
1               5                 10              15

Leu Pro Ile Ser Met Lys Ile Phe Met Tyr Leu Leu Thr Val Phe Leu
                20              25              30

Ile Thr Gln Met Ile Gly Ser Ala Leu Phe Ala Val Tyr Leu His Arg
            35              40              45
```

```
     Arg Leu Asp Lys Ile Glu Asp Glu Arg Asn Leu His Glu Asp Phe Val
         50                  55                  60

 5   Phe Met Lys Thr Ile Gln Arg Cys Asn Thr Gly Glu Arg Ser Leu Ser
      65                  70                  75                  80

     Leu Leu Asn Cys Glu Glu Ile Lys Ser Gln Phe Glu Gly Phe Val Lys
                     85                  90                  95

10   Asp Ile Met Leu Asn Lys Glu Glu Thr Lys Lys Glu Asn Ser Phe Glu
                    100                 105                 110

     Met Gln Lys Gly Asp Gln Asn Pro Gln Ile Ala Ala His Val Ile Ser
             115                 120                 125
15
     Glu Ala Ser Ser Lys Thr Thr Ser Val Leu Gln Trp Ala Glu Lys Gly
         130                 135                 140

     Tyr Tyr Thr Met Ser Asn Asn Leu Val Thr Leu Glu Asn Gly Lys Gln
20   145                 150                 155                 160

     Leu Thr Val Lys Arg Gln Gly Leu Tyr Tyr Ile Tyr Ala Gln Val Thr
                    165                 170                 175

25   Phe Cys Ser Asn Arg Glu Ala Ser Ser Gln Ala Pro Phe Ile Ala Ser
                    180                 185                 190

     Leu Cys Leu Lys Ser Pro Gly Arg Phe Glu Arg Ile Leu Leu Arg Ala
             195                 200                 205
30
     Ala Asn Thr His Ser Ser Ala Lys Pro Cys Gly Gln Gln Ser Ile His
         210                 215                 220

     Leu Gly Gly Val Phe Glu Leu Gln Pro Gly Ala Ser Val Phe Val Asn
35   225                 230                 235                 240

     Val Thr Asp Pro Ser Gln Val Ser His Gly Thr Gly Phe Thr Ser Phe
                    245                 250                 255

40   Gly Leu Leu Lys Leu
                    260
```

```
45   (2)  INFORMATION FOR SEQ ID NO:13:

          (i)  SEQUENCE CHARACTERISTICS:
               (A)  LENGTH: 73 base pairs
               (B)  TYPE: nucleic acid
               (C)  STRANDEDNESS: single
50             (D)  TOPOLOGY: linear

          (ii)  MOLECULE TYPE: cDNA to mRNA

          (iii)  HYPOTHETICAL: NO
55
          (iv)  ANTI-SENSE: NO


60        (xi)  SEQUENCE DESCRIPTION: SEQ ID NO:13:
```

TGGTGGCGGA GGGTCAGGCG GAGGTGGGTC CGGAGGCGGG GGTTCAAGTT CTGACAAGAT
60

AGAAGATGAA AGG
73

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGCCGCTCAG AGTTTGAGTA A
21

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1425 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA to mRNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vii) IMMEDIATE SOURCE:
        (B) CLONE: Human CD40-L/FC2 (soluble CD40-L)

    (ix) FEATURE:
        (A) NAME/KEY: CDS
        (B) LOCATION: 4..1422

    (ix) FEATURE:
        (A) NAME/KEY: mat_peptide
        (B) LOCATION: 79..1422

    (ix) FEATURE:
        (A) NAME/KEY: sig_peptide
        (B) LOCATION: 4..78

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
        TAT ATG TTC CAT GTT TCT TTT AGA TAT ATC TTT GGA ATT CCT CCA CTG
        48
            Met Phe His Val Ser Phe Arg Tyr Ile Phe Gly Ile Pro Pro Leu
            -25             -20             -15
5
        ATC CTT GTT CTG CTG CCT GTC ACT AGC TCT GAC TAC AAA GAT GAC GAT
        96
        Ile Leu Val Leu Leu Pro Val Thr Ser Ser Asp Tyr Lys Asp Asp Asp
        -10             -5              1               5
10
        GAT AAA AGA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA
        144
        Asp Lys Arg Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala
                    10              15              20
15
        CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC
        192
        Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro
                25              30              35
20
        AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG
        240
        Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
            40              45              50
25
        GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG
        288
        Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val
        55              60              65              70
30
        GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG
        336
        Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
                    75              80              85
35
        TAC AAC AGC ACG TAC CGG GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG
        384
        Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
                    90              95              100
40
        GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC
        432
        Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
            105             110             115
45
        CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC
        480
        Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
            120             125             130
50
        CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC
        528
        Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr
        135             140             145             150
55
        AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC
        576
        Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
                    155             160             165
60
```

```
      GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC
      624
      Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr
                  170             175                 180

 5    AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC
      672
      Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr
          185                 190                 195

10    AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC
      720
      Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe
              200             205                 210

15    TCA TGC TCC GTG ATG CAT GGT GGC GGA GGG TCA GGC GGA GGT GGG TCC
      768
      Ser Cys Ser Val Met His Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
      215                 220                 225                 230

20    GGA GGC GGG GGT TCA AGT TCT GAC AAG ATA GAA GAT GAA AGG AAT CTT
      816
      Gly Gly Gly Gly Ser Ser Ser Asp Lys Ile Glu Asp Glu Arg Asn Leu
                      235                 240                 245

25    CAT GAA GAT TTT GTA TTC ATG AAA ACG ATA CAG AGA TGC AAC ACA GGA
      864
      His Glu Asp Phe Val Phe Met Lys Thr Ile Gln Arg Cys Asn Thr Gly
                  250                 255                 260

30    GAA AGA TCC TTA TCC TTA CTG AAC TGT GAG GAG ATT AAA AGC CAG TTT
      912
      Glu Arg Ser Leu Ser Leu Leu Asn Cys Glu Glu Ile Lys Ser Gln Phe
              265                 270                 275

35    GAA GGC TTT GTG AAG GAT ATA ATG TTA AAC AAA GAG GAG ACG AAG AAA
      960
      Glu Gly Phe Val Lys Asp Ile Met Leu Asn Lys Glu Glu Thr Lys Lys
          280                 285                 290

40    GAA AAC AGC TTT GAA ATG CAA AAA GGT GAT CAG AAT CCT CAA ATT GCG
      1008
      Glu Asn Ser Phe Glu Met Gln Lys Gly Asp Gln Asn Pro Gln Ile Ala
      295                 300                 305                 310

45    GCA CAT GTC ATA AGT GAG GCC AGC AGT AAA ACA ACA TCT GTG TTA CAG
      1056
      Ala His Val Ile Ser Glu Ala Ser Ser Lys Thr Thr Ser Val Leu Gln
                      315                 320                 325

50    TGG GCT GAA AAA GGA TAC TAC ACC ATG AGC AAC AAC TTG GTA ACC CTG
      1104
      Trp Ala Glu Lys Gly Tyr Tyr Thr Met Ser Asn Asn Leu Val Thr Leu
                  330                 335                 340

55    GAA AAT GGG AAA CAG CTG ACC GTT AAA AGA CAA GGA CTC TAT TAT ATC
      1152
      Glu Asn Gly Lys Gln Leu Thr Val Lys Arg Gln Gly Leu Tyr Tyr Ile
                  345                 350                 355

60
```

```
TAT GCC CAA GTC ACC TTC TGT TCC AAT CGG GAA GCT TCG AGT CAA GCT
1200
Tyr Ala Gln Val Thr Phe Cys Ser Asn Arg Glu Ala Ser Ser Gln Ala
    360                 365             370

CCA TTT ATA GCC AGC CTC TGC CTA AAG TCC CCC GGT AGA TTC GAG AGA
1248
Pro Phe Ile Ala Ser Leu Cys Leu Lys Ser Pro Gly Arg Phe Glu Arg
375                 380             385                 390

ATC TTA CTC AGA GCT GCA AAT ACC CAC AGT TCC GCC AAA CCT TGC GGG
1296
Ile Leu Leu Arg Ala Ala Asn Thr His Ser Ser Ala Lys Pro Cys Gly
                395             400             405

CAA CAA TCC ATT CAC TTG GGA GGA GTA TTT GAA TTG CAA CCA GGT GCT
1344
Gln Gln Ser Ile His Leu Gly Gly Val Phe Glu Leu Gln Pro Gly Ala
            410             415             420

TCG GTG TTT GTC AAT GTG ACT GAT CCA AGC CAA GTG AGC CAT GGC ACT
1392
Ser Val Phe Val Asn Val Thr Asp Pro Ser Gln Val Ser His Gly Thr
        425             430             435

GGC TTC ACG TCC TTT GGC TTA CTC AAA CTC TGA
1425
Gly Phe Thr Ser Phe Gly Leu Leu Lys Leu
    440             445
```

(2) INFORMATION FOR SEQ ID NO:16:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 473 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: protein

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Met Phe His Val Ser Phe Arg Tyr Ile Phe Gly Ile Pro Pro Leu Ile
-25                 -20             -15                 -10

Leu Val Leu Leu Pro Val Thr Ser Ser Asp Tyr Lys Asp Asp Asp Asp
                -5                  1               5

Lys Arg Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro
        10              15              20

Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
    25              30              35

Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
    40              45              50              55

Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
                60              65              70

Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr
```

	75						80					85		

Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
90   95   100

5   Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
105   110   115

10   Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg
120   125   130   135

     Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys
140   145   150

15   Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
155   160   165

     Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
170   175   180

20   Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
185   190   195

25   Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
200   205   210   215

     Cys Ser Val Met His Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
220   225   230

30   Gly Gly Gly Ser Ser Ser Asp Lys Ile Glu Asp Glu Arg Asn Leu His
235   240   245

     Glu Asp Phe Val Phe Met Lys Thr Ile Gln Arg Cys Asn Thr Gly Glu
250   255   260

35   Arg Ser Leu Ser Leu Leu Asn Cys Glu Glu Ile Lys Ser Gln Phe Glu
265   270   275

40   Gly Phe Val Lys Asp Ile Met Leu Asn Lys Glu Glu Thr Lys Lys Glu
280   285   290   295

     Asn Ser Phe Glu Met Gln Lys Gly Asp Gln Asn Pro Gln Ile Ala Ala
300   305   310

45   His Val Ile Ser Glu Ala Ser Ser Lys Thr Thr Ser Val Leu Gln Trp
315   320   325

     Ala Glu Lys Gly Tyr Tyr Thr Met Ser Asn Asn Leu Val Thr Leu Glu
330   335   340

50   Asn Gly Lys Gln Leu Thr Val Lys Arg Gln Gly Leu Tyr Tyr Ile Tyr
345   350   355

55   Ala Gln Val Thr Phe Cys Ser Asn Arg Glu Ala Ser Ser Gln Ala Pro
360   365   370   375

     Phe Ile Ala Ser Leu Cys Leu Lys Ser Pro Gly Arg Phe Glu Arg Ile
380   385   390

60   Leu Leu Arg Ala Ala Asn Thr His Ser Ser Ala Lys Pro Cys Gly Gln
395   400   405

59

```
Gln Ser Ile His Leu Gly Gly Val Phe Glu Leu Gln Pro Gly Ala Ser
        410             415             420

Val Phe Val Asn Val Thr Asp Pro Ser Gln Val Ser His Gly Thr Gly
        425             430             435

Phe Thr Ser Phe Gly Leu Leu Lys Leu
440             445
```

(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 33 amino acids
       (B) TYPE: amino acid
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Arg Met Lys Gln Ile Glu Asp Lys Ile Glu Glu Ile Leu Ser Lys Ile
1           5               10              15

Tyr His Ile Glu Asn Glu Ile Ala Arg Ile Lys Lys Leu Ile Gly Glu
            20              25              30

Arg
```

CLAIMS

What is claimed is:

1. An isolated DNA sequence encoding a CD40-L polypeptide that binds to CD40, wherein the DNA sequence is selected from the group consisting of:

(a) nucleotides 46 through 828, 184 through 828, or 193 through 762 of SEQ ID NO:11;

(b) DNA sequences which detectably hybridize to one of the sequences of (a) or their complementary strands under moderate stringency conditions; and

(c) DNA sequences which, due to degeneracy of the genetic code, encode a polypeptide encoded by any of the foregoing DNA sequences.

2. An isolated DNA sequence according to claim 1 encoding a soluble CD40-L polypeptide.

3. A recombinant expression vector comprising a DNA sequence according to claim 1.

4. A recombinant expression vector comprising a DNA sequence according to claim 2.

5. A host cell transformed or transfected with an expression vector according to claim 3.

6. A host cell transformed or transfected with an expression vector according to claim 4.

7. A process for preparing a CD40-L polypeptide, comprising culturing a host cell according to claim 5 under conditions promoting expression and recovering CD40-L polypeptide from the culture.

8. A process for preparing a CD40-L polypeptide, comprising culturing a host cell according to claim 6 under conditions promoting expression and recovering CD40-L polypeptide from the culture.

9. A purified biologically active CD40-L polypeptide composition comprising a sequence of amino acids encoded by a nucleotide sequence according to claim 1.

10. A purified biologically active CD40-L polypeptide composition according to claim 9, consisting essentially of human CD40-L.

11. A purified biologically active CD40-L polypeptide composition according to claim 10, selected from the group consisting:

(a) the polypeptide defined by amino acids 1 through 261, inclusive, of the sequence set forth in SEQ ID NO:11;

(b) the polypeptide defined by amino acids 47 through 261, inclusive, of the sequence set forth in SEQ ID NO:11; and

(c) a polypeptide defined by a sequence beginning with an amino acid in the sequence between amino acid 47 and amino acid 51, inclusive, through and including an amino acid in the sequence between amino acid 239 and amino acid 261, inclusive, of the sequence set forth in SEQ ID NO:11.

12. A purified biologically active CD40-L polypeptide composition according to claim 11, wherein the CD40-L is a soluble CD40-L.

13. A soluble CD40-L according to claim 12, wherein the soluble CD40-1 comprises amino acids 51 through 261 of SEQ ID NO:11.

14. A soluble CD40-L according to claim 13 wherein the CD40-L is an oligomer comprising two or more CD40-L extracellular regions.

15. A method of treating allergy, an allergic reaction, lupus, rheumatoid arthritis or graft versus host disease comprising administering an effective amount of a CD40 antagonist selected from the group consisting of a soluble CD40 protein, a CD40 fusion protein, a soluble monomeric CD40-L polypeptide, and combinations thereof.

16. A method of augmenting a vaccine response with an adjuvant, wherein the adjuvant is selected from the group consisting of a membrane-bound CD40-L polypeptide, oligomeric soluble CD40-L polypeptides, and combinations thereof.

17. A method for stimulating hybridoma cells to increase monoclonal antibody secretion, comprising administering an effective amount of a CD40 agonist selected

from the group consisting of CD40-L, membrane-bound CD40-L and oligomeric CD40-L.

18. An antibody immunoreactive with CD40-L or a CD40-L immunogen.

5

19. An antibody according to claim 16 which is a monoclonal antibody.

20. An antisense or sense oligonucleotide that can inhibit transcription or translation of CD40-L, comprising a sequence of at least about 12 nucleotides
10 corresponding to a DNA sequence according to claim 1 or its DNA or RNA complement.

FIG. 1

```
   1  CTTTCAGTCAGCATGATAGAAACATACAGCCAACCTTCCCCCAGATCCGTGGCAACTGGA    60
   1                 MetIleGluThrTyrSerGlnProSerProArgSerValAlaThrGly   16

  61  CTTCCAGCGAGCATGAAGATTTTTATGTATTTACTTACTGTTTTCCTTATCACCCAAATG   120
  17  LeuProAlaSerMetLysIlePheMetTyrLeuLeuThrValPheLeuIleThrGlnMet   36

 121  ATTGGATCTGTGCTTTTTGCTGTGTATCTTCATAGAAGATTGGATAAGGTCGAAGAGGAA   180
  37  IleGlySerValLeuPheAlaValTyrLeuHisArgArgLeuAspLysValGluGluGlu   56

 181  GTAAACCTTCATGAAGATTTTGTATTCATAAAAAAAGCTAAAGAGATGCAACAAAGGAGAA   240
  57  ValAsnLeuHisGluAspPheValPheIleLysLysLeuLysArgCysAsnLysGlyGlu   76

 241  GGATCTTTATCCTTGCTGAACTGTGAGGAGATGAGAAGGCAATTTGAAGACCTTGTCAAG   300
  77  GlySerLeuSerLeuLeuAsnCysGluGluMetArgArgGlnPheGluAspLeuValLys   96

 301  GATATAACGTTAAACAAAGAAGAGAAAAAGAAACAGCTTTGAAATGCAAAGAGGTGAT    360
  97  AspIleThrLeuAsnLysGluGluLysLysGluAsnSerPheGluMetGlnArgGlyAsp   116

 361  GAGGATCCTCAAATTGCAGCACACGTTGTAAGCGAAGCCAACAGTAATGCAGCATCCGTT   420
 117  GluAspProGlnIleAlaAlaHisValValSerGluAlaAsnSerAsnAlaAlaSerVal   136

 421  CTACAGTGGGCCAAGAAAGGATATTATACCATGAAAAGCAACTTGGTAATGCTTGAAAAT   480
 137  LeuGlnTrpAlaLysLysGlyTyrTyrThrMetLysSerAsnLeuValMetLeuGluAsn   156

 481  GGGAAACAGCTGACGGTTAAAAGAGAAGGACTCTATTATGTCTACACTCAAGTCACCTTC   540
 157  GlyLysGlnLeuThrValLysArgGluGlyLeuTyrTyrValTyrThrGlnValThrPhe   176

 541  TGCTCTAATCGGGAGCCTTCGAGTCAACGCCCATTCATCGTCGGCCTCTGGCTGAAGCCC   600
 177  CysSerAsnArgGluProSerSerGlnArgProPheIleValGlyLeuTrpLeuLysPro   196

 601  AGCATTGGATCTGAGAGAATCTTACTCAAGGCGGCAAATACCCACAGTTCCTCCCAGCTT   660
 197  SerIleGlySerGluArgIleLeuLeuLysAlaAlaAsnThrHisSerSerSerGlnLeu   216

 661  TGCGAGCAGCAGTCTGTTCACTTGGGCGGAGTGTTTGAATTACAAGCTGGTGCTTCTGTG   720
 217  CysGluGlnGlnSerValHisLeuGlyGlyValPheGluLeuGlnAlaGlyAlaSerVal   236

 721  TTTGTCAACGTGACTGAAGCAAGCCAAGTGATCCACAGAGTTGGCTTCTCATCTTTTGGC   780
 237  PheValAsnValThrGluAlaSerGlnValIleHisArgValGlyPheSerSerPheGly   256

 781  TTACTCAAACTCTGAACAGTGCGCTGCCCTAGGCTGCAGCAGGGCTGATGCTGGCAGTCT   840
 257  LeuLeuLysLeu                                                  260

 841  cCCCTATACAcCAAGTCAGTTAGGcCCTcCCCTGTGTTGAACTgCCTATTTATAACCCTA   900

 901  GGATCCTCCTCATGGAGAAcTATTTATTATGTACCCCCAAGGCACATAGAGCTGGAATAA   960

 961  GAGAATTACAGGGCAGGCAAAAATCCCAAGGGACCCTGCTCCCTAAGAACTTACAATCTG  1020

1021  AAACAGCAACCCCACTGATTCAGACAACCAGAAAAGACAAAGCCATAATACACAGATGAC  1080

1081  AGAGCTCTGATGAAACAACAGATAACTAATGAGCACAGTTTTGTTGTTTTATGGGTGTGT  1140

1141  CGTTCAATGGACAGTGTACTTGACTTACCAGGGAAGATGCAGAAGGGCAACTGTGAGCCT  1200

1201  CAGCTCACAATCTGTTATGGTTGACCTGGGCTCCCTGCGGCCCTAGTAGG  1250
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  1   TGCCACCTTCTCTGCCAGAAGATACCATTTCAACTTTAACACAGCATGATCGAAACATAC     60
                                                   MetIleGluThrTyr        5
  1

 61   AACCAAACTTCTCCCCGATCTGCGGCCACTGGACTGCCCATCAGCATGAAAATTTTTATG    120
  6   AsnGlnThrSerProArgSerAlaAlaThrGlyLeuProIleSerMetLysIlePheMet    25

121   TATTTACTTACTGTTTTTCTTATCACCCAGATGATTGGGTCAGCACTTTTTGCTGTGTAT    180
 26   TyrLeuLeuThrValPheLeuIleThrGlnMetIleGlySerAlaLeuPheAlaValTyr    45

181   CTTCATAGAAGGTTGGACAAGATAGAAGATGAAAGGAATCTTCATGAAGATTTTGTATTC    240
 46   LeuHisArgArgLeuAspLysIleGluAspGluArgAsnLeuHisGluAspPheValPhe    65

241   ATGAAAACGATACAGAGATGCAACACAGGAGAAAGATCCTTATCCTTACTGAACTGTGAG    300
 66   MetLysThrIleGlnArgCysAsnThrGlyGluArgSerLeuSerLeuLeuAsnCysGlu    85

301   GAGATTAAAAGCCAGTTTGAAGGCTTTGTGAAGGATATAATGTTAAACAAAGAGGAGACG    360
 86   GluIleLysSerGlnPheGluGlyPheValLysAspIleMetLeuAsnLysGluGluThr    105

361   AAGAAAGAAAACAGCTTTGAAATGCAAAAAGGTGATCAGAATCCTCAAATTGCGGCACAT    420
106   LysLysGluAsnSerPheGluMetGlnLysGlyAspGlnAsnProGlnIleAlaAlaHis    125

421   GTCATAAGTGAGGCCAGCAGTAAAACAACATCTGTGTTACAGTGGGCTGAAAAAGGATAC    480
126   ValIleSerGluAlaSerSerLysThrThrSerValLeuGlnTrpAlaAspLysGlyTyr    145

481   TACACCATGAGCAACAACTTGGTAACCCTGGAAAATGGGAAACAGCTGACCGTTAAAAGA    540
146   TyrThrMetSerAsnAsnLeuValThrLeuGluAsnGlyLysGlnLeuThrValLysArg    165

541   CAAGGACTCTATTATATCTATGCCCAAGTCACCTTCTGTTCCAATCGGGAAGCTTCGAGT    600
166   GlnGlyLeuTyrTyrIleTyrAlaGlnValThrPheCysSerAsnArgGluAlaSerSer    185

601   CAAGCTCCATTTATAGCCAGCCTCTGCCTAAAGTCCCCCGGTAGATTCGAGAGAATCTTA    660
186   GlnAlaProPheIleAlaSerLeuCysLeuLysSerProGlyArgPheGluArgIleLeu    205

661   CTCAGAGCTGCAAATACCCACAGTTCCGCCAAACCTTGCGGGCAACAATCCATTCACTTG    720
206   LeuArgAlaAlaAsnThrHisSerSerAlaLysProCysGlyGlnGlnSerIleHisLeu    225

721   GGAGGAGTATTTGAATTGCAACCAGGTGCTTCGGTGTTTGTCAATGTGACTGATCCAAGC    780
226   GlyGlyValPheGluLeuGlnProGlyAlaSerValPheValAsnValThrAspProSer    245

781   CAAGTGAGCCATGGCACTGGCTTCACGTCCTTTGGCTTACTCAAACTCTGAACAGTGTCA    840
246   GlnValSerHisGlyThrGlyPheThrSerPheGlyLeuLeuLysLeu                 261

841   CCTTGCAGGCTGTGGTGGAGCTGACGCTGGGAGTCTTCATAATACAGCACAGCGGTTAAGC    900

901   CCACCCCCTGTTAACTGCCTATTTATAACCCTAGGATCCTCCTTATGGAGAACTATTTAT    960
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FIG. 3

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1    MIETYNQTSPRSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHRRL   50
     |||| ·  · ||| |||| ·|||||||||||||||||||||· |||||||||
5    MIETYSQPSPRSVATGLPASMKIFMYLLTVFLITQMIGSVLFAVYLHRRL   54

51   DKIEDERNLHEDFVFMKTIQRCNTGERSLSLLNCEEIKSQFEGFVKDIML  100
     ||· ·: |||||||| ·· |·||·|| ||||||||||· ·: ||· | ||
55   DKVEEVNLHEDFVFIKKLKRCNKGEGSLSLLNCEEMRRQFEDLVKDITL   104

101  NKEETKKENSFEMQKGDQNPQIAAHVISEASSKTTSVLQWAEKGYYTMSN  150
     |||| ·||||||||· ||· |||||||·||| · ·  |||||· ||||||· ·
105  NKEE.KKENSFEMQRGDEDPQIAAHVVSEANSNAASVLQWAKKGYYTMKS   153

151  NLVTLENGKQLTVKRQGLYYIYAQVTFCSNREASSQAPFIASLCLKSPGR  200
     |||·||||||||||| ·|||·|·· |||||||||· :||· ||· ·  ·|·|·
154  NLVMLENGKQLTVKREGLYYVYTQVTFCSNREPSSQRPFIVGLWLKPSIG   203

201  FERILLRAANTHSSAKPCGQQSIHLGGVFELQPGASVFVNVTDPSQVSHG  250
     ||||| ·|||||||· ·|·|||||·||||||||· ||||||||· · | ||·
204  SERILLKAANTHSSSQLCEQQSVHLGGVFELQAGASVFVNVTEASQVIHR   253

251  TGFTSFGLLKL  261
     · || ·|||||||
254  VGFSSFGLLKL  264
```

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F I G . 4A

10E5 E- + CV1's

Number of CV1's

- ○- HAV EO
- ●- CD40L

F I G . 4B

10E5 E- + IL-4 + CV1's

Number of CV1's

- ○- HAV EO
- ●- CD40L
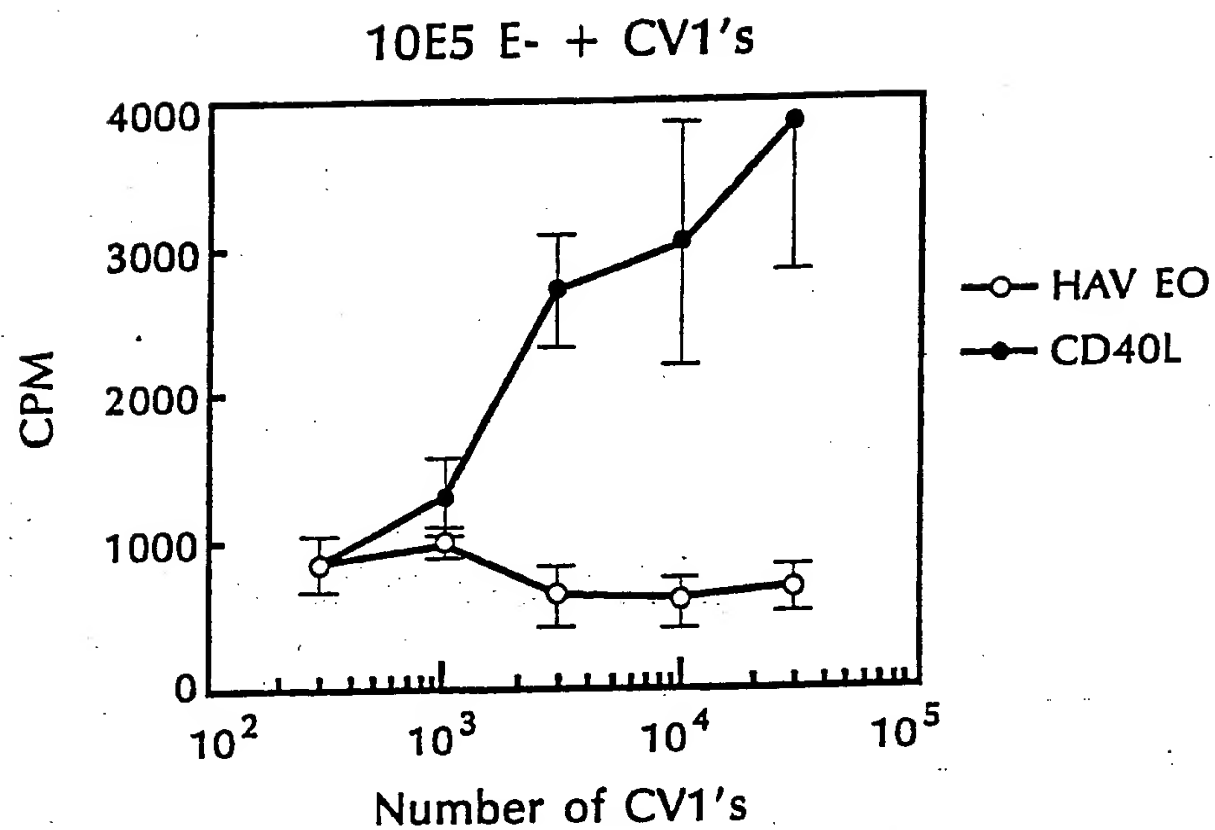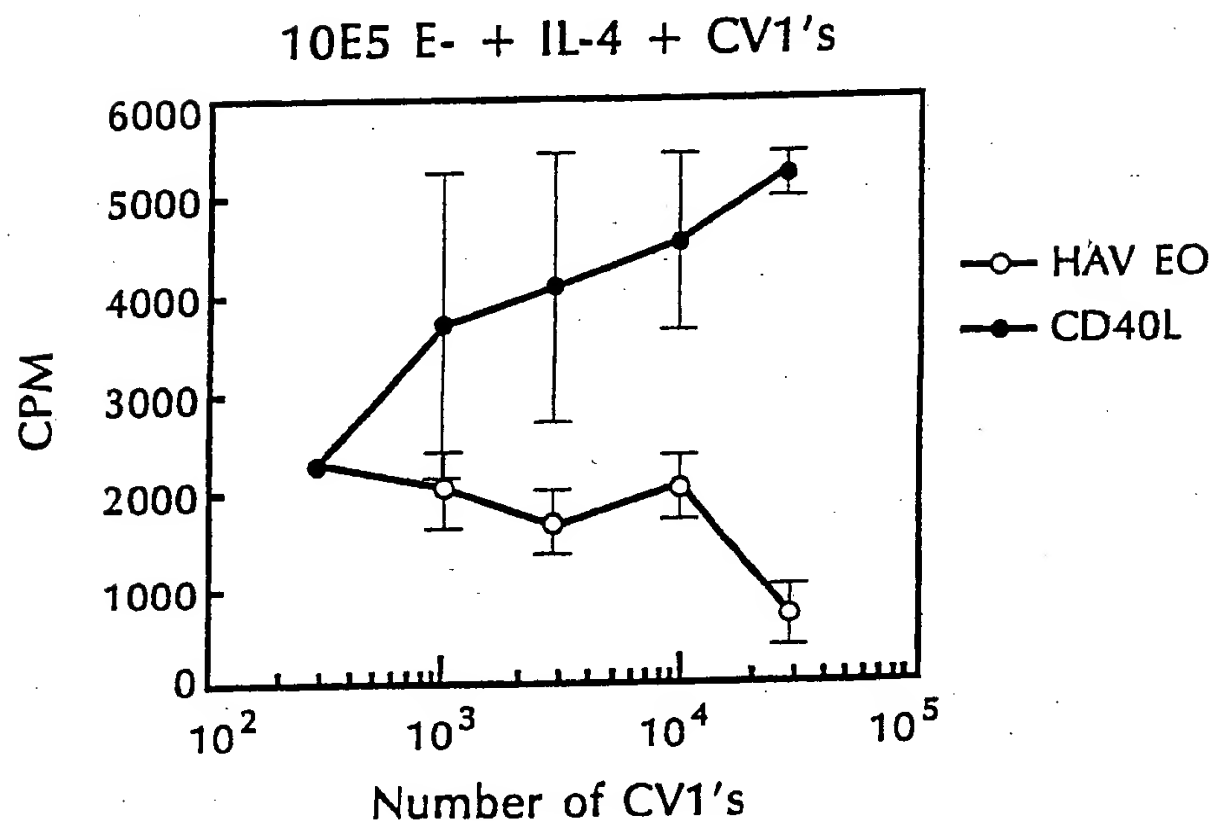
SUBSTITUTE SHEET

FIG. 5

PB E + IL-4 + CV1 d7 Proliferation
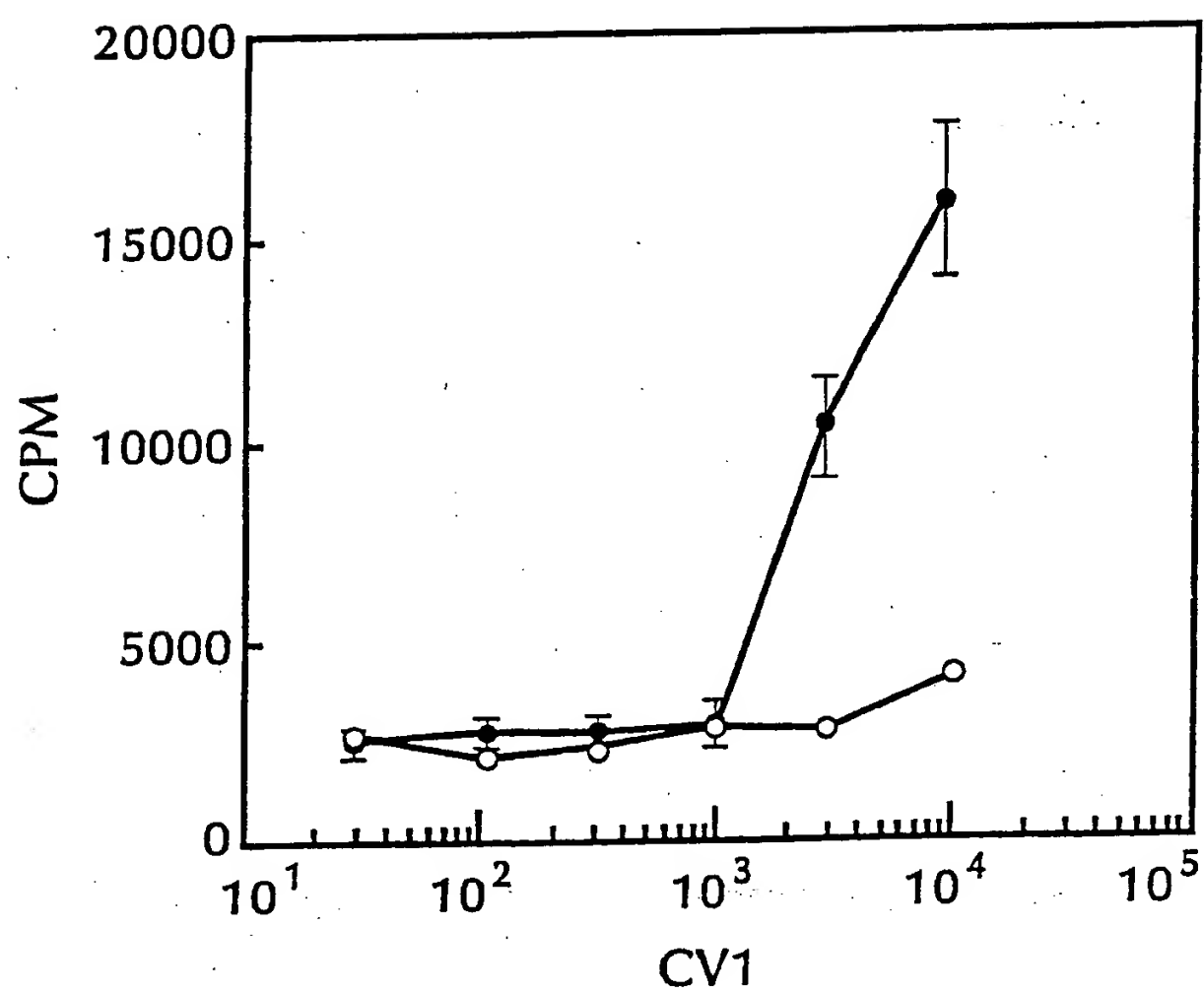
—○— HAV
—●— CD40L

FIG. 6

S.CD23 in Day 6 Cultures S/N:
10E5 E-/Well, IMDM + IL-4

Legend inside chart:
▨ CV1 HAV EO
■ CV1 CD40L

MED. 4.7
IL-4 2.9
IL-4 + G28.5 >600

Y-axis: IgE ng/ml
X-axis: Number CV1's (300, 1000, 3000, 10000, 30000)
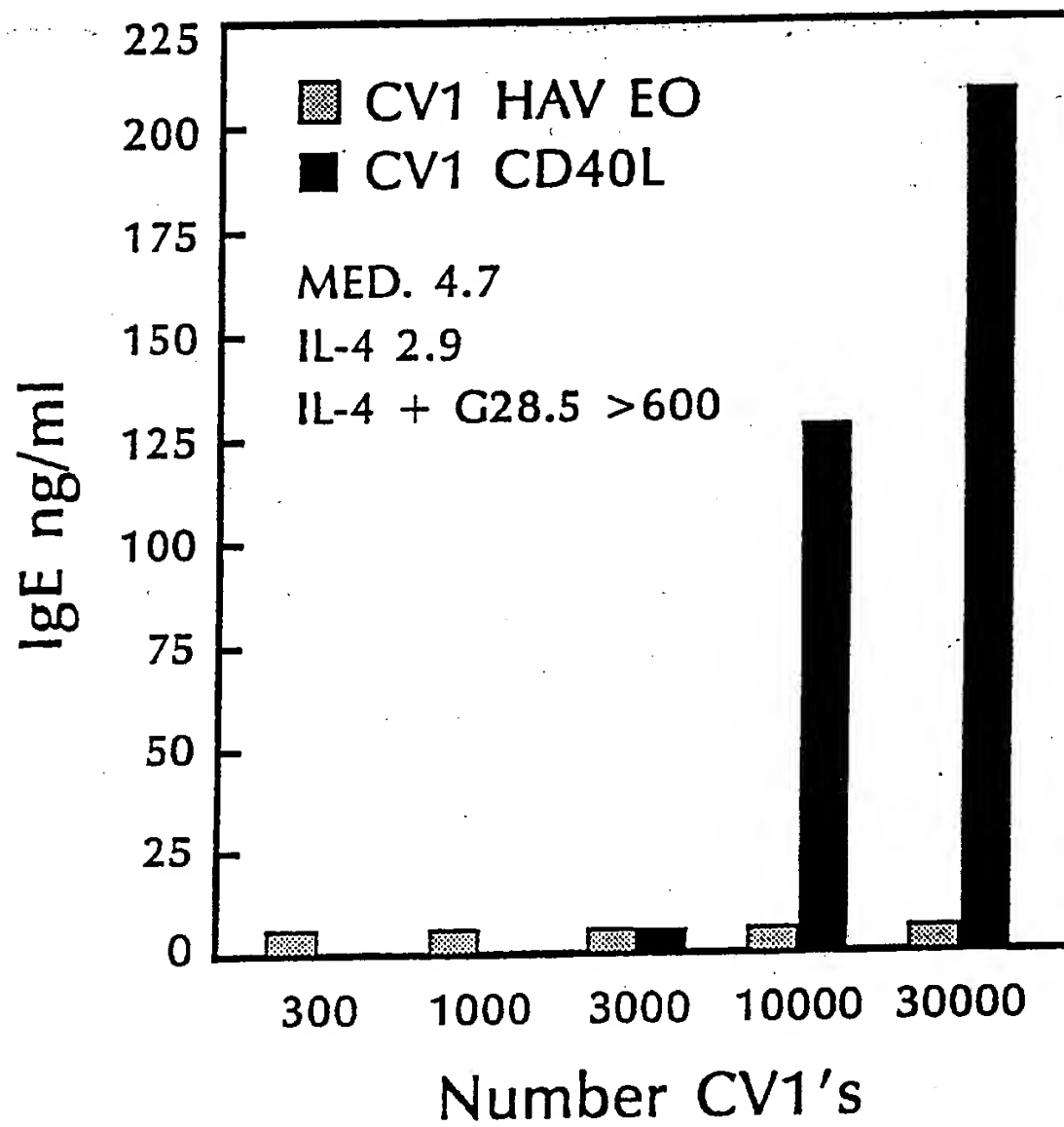
FIG. 7

S.CD23 in Day 6 Cultures S/N:
10E5 E-/Well, IMDM + IL-4
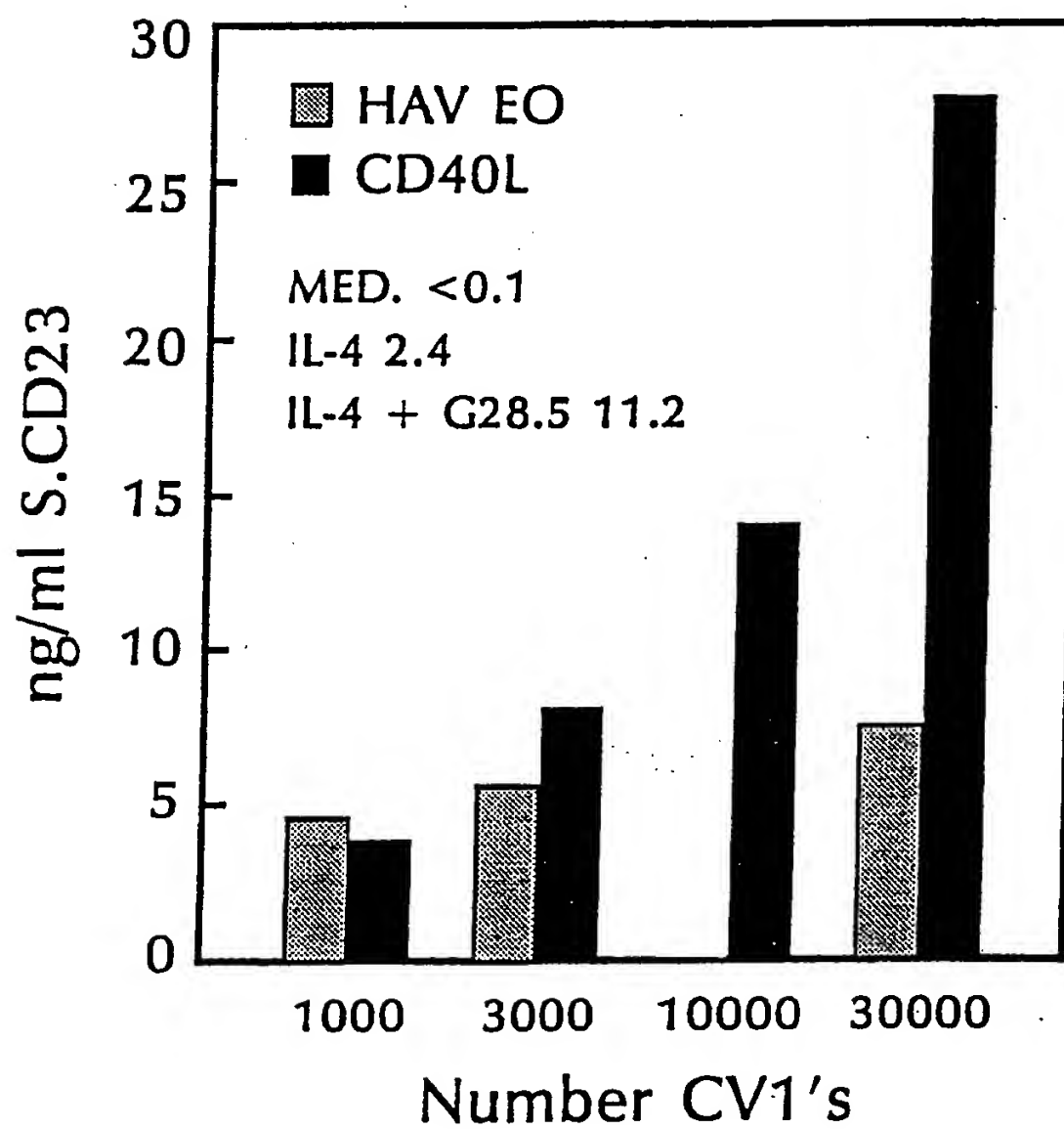
FIG. 8

Induction of B Cell Proliferation by
CD40 Ligand Expressing CV-1 Cells (fixed)
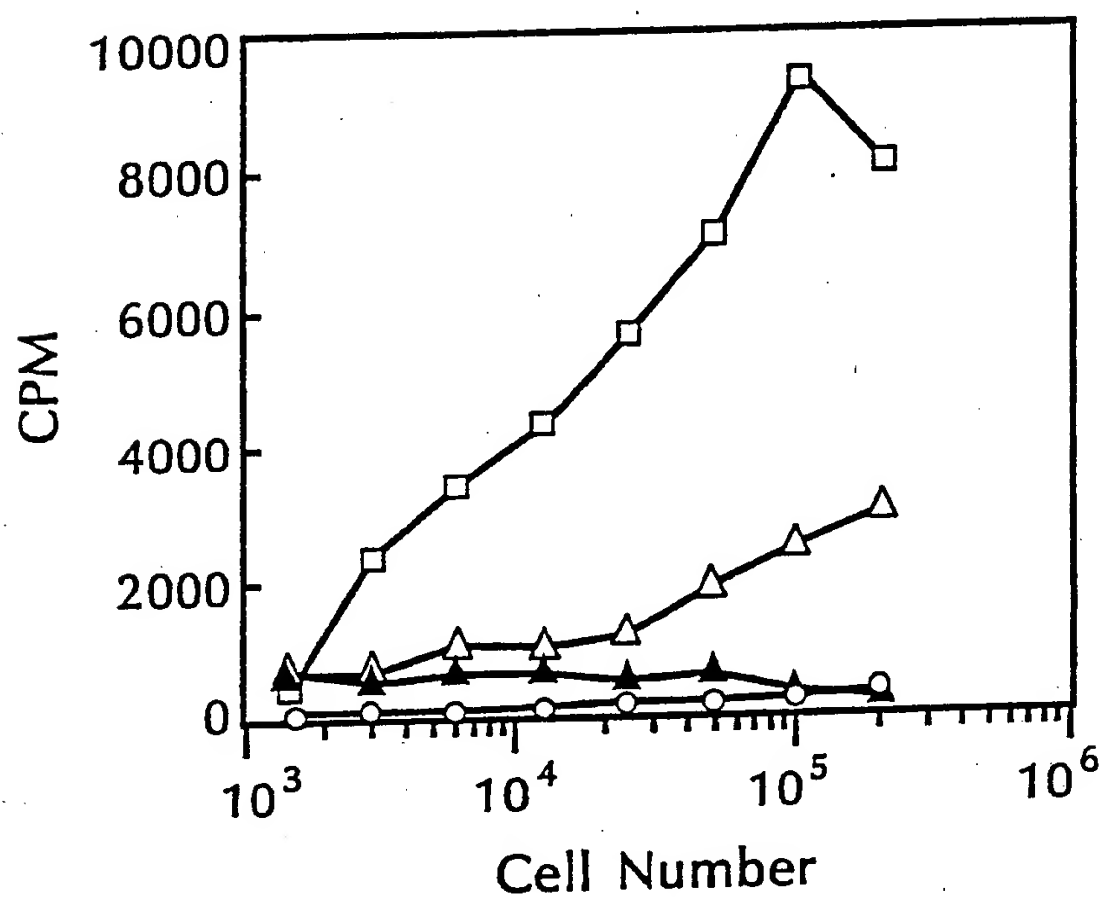
—□— CD40L

—△— 7A1

—▲— HAV EO

—○— 40.9

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FIG. 9

Induction of Anti-SRBC PFC by EL4 40.9 and 7A1 Th1 Cells (Fixed)

—□— 7A1
—○— 40.9
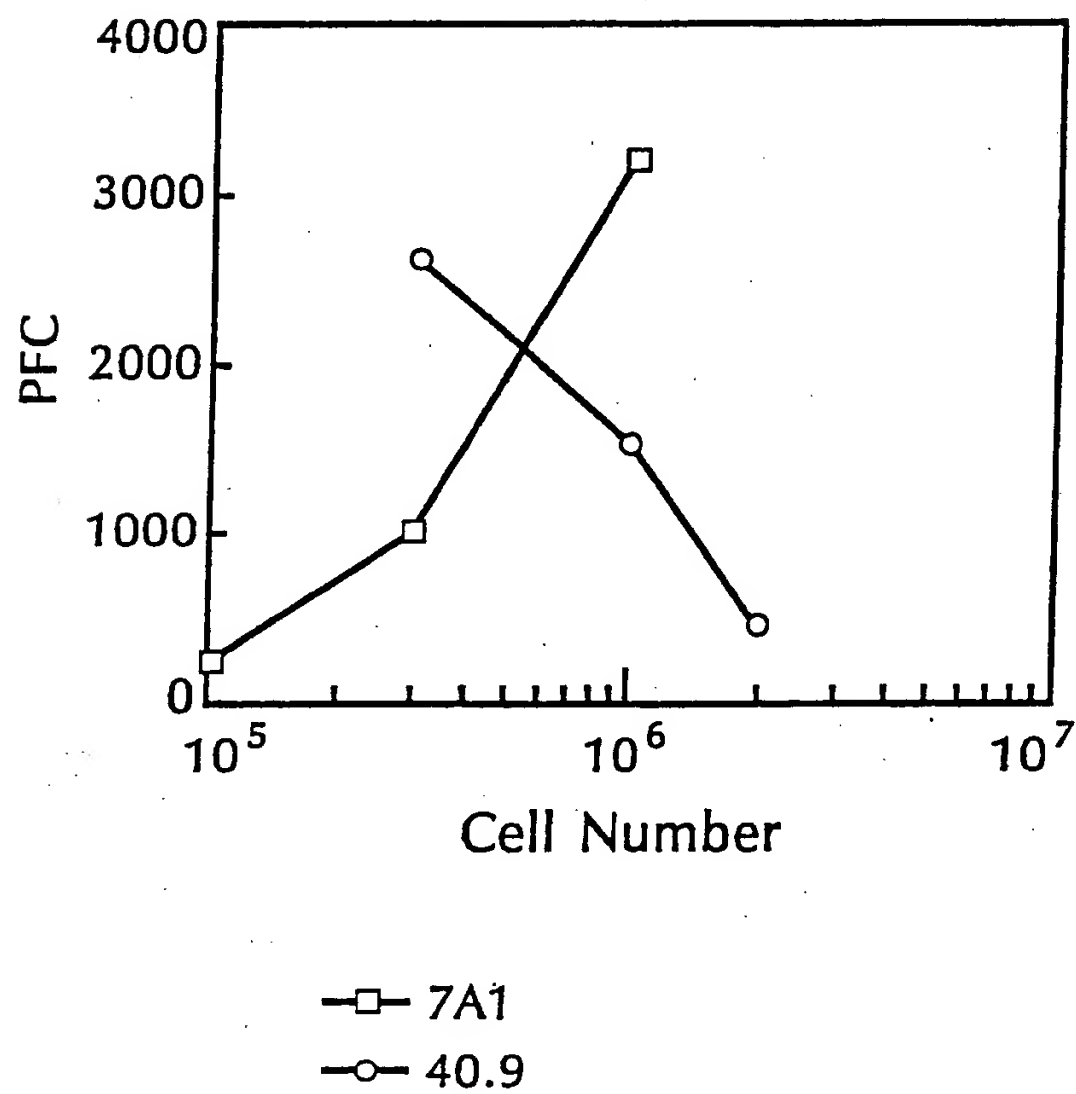
FIG. 10

Induction of B Cell Proliferation by CD40 Ligand Expressing CV-1 Cells
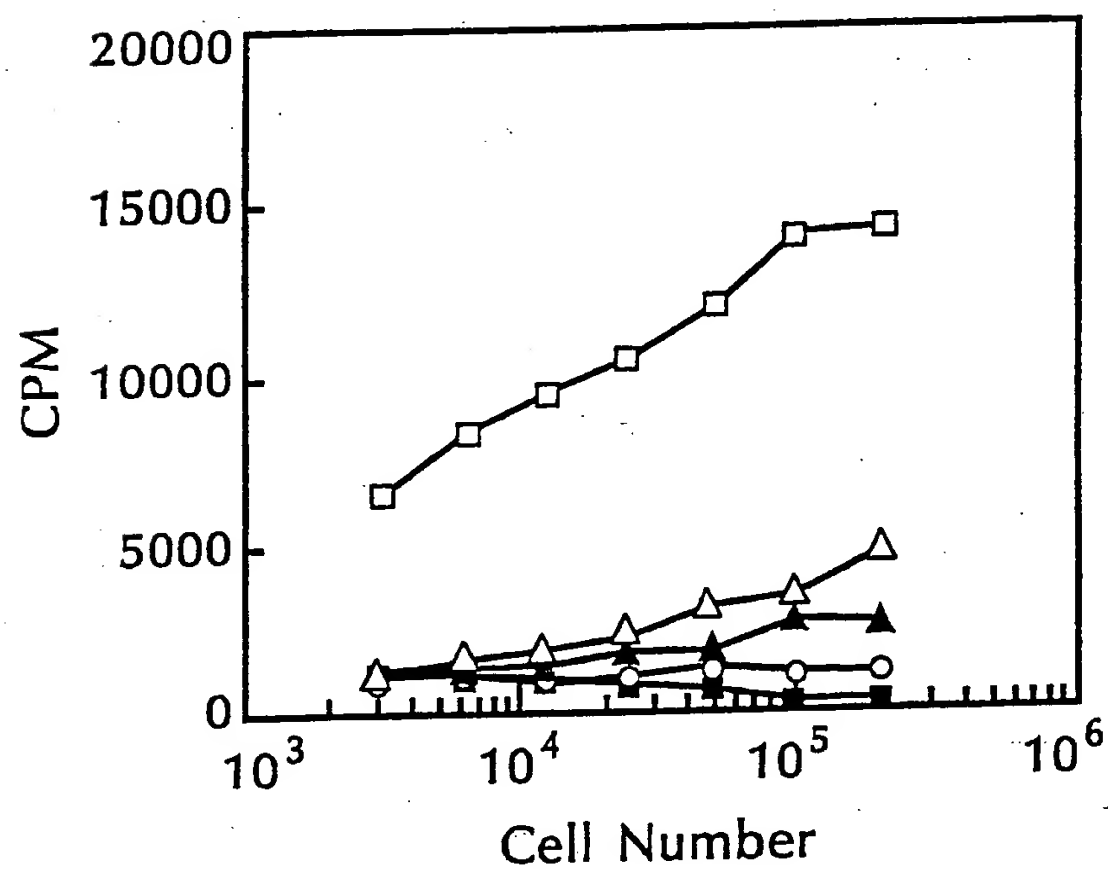
--□-- CD40L
--△-- 7A1
--■-- HAV EO
--▲-- 7A1 + CD40Fc
--○-- CD40Fc

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FIG. 11

Induction of Anti-SRBC PFC by CD40
Ligand Expressing CV-1 Cells (fixed)

PFC or % Recovery (x10)
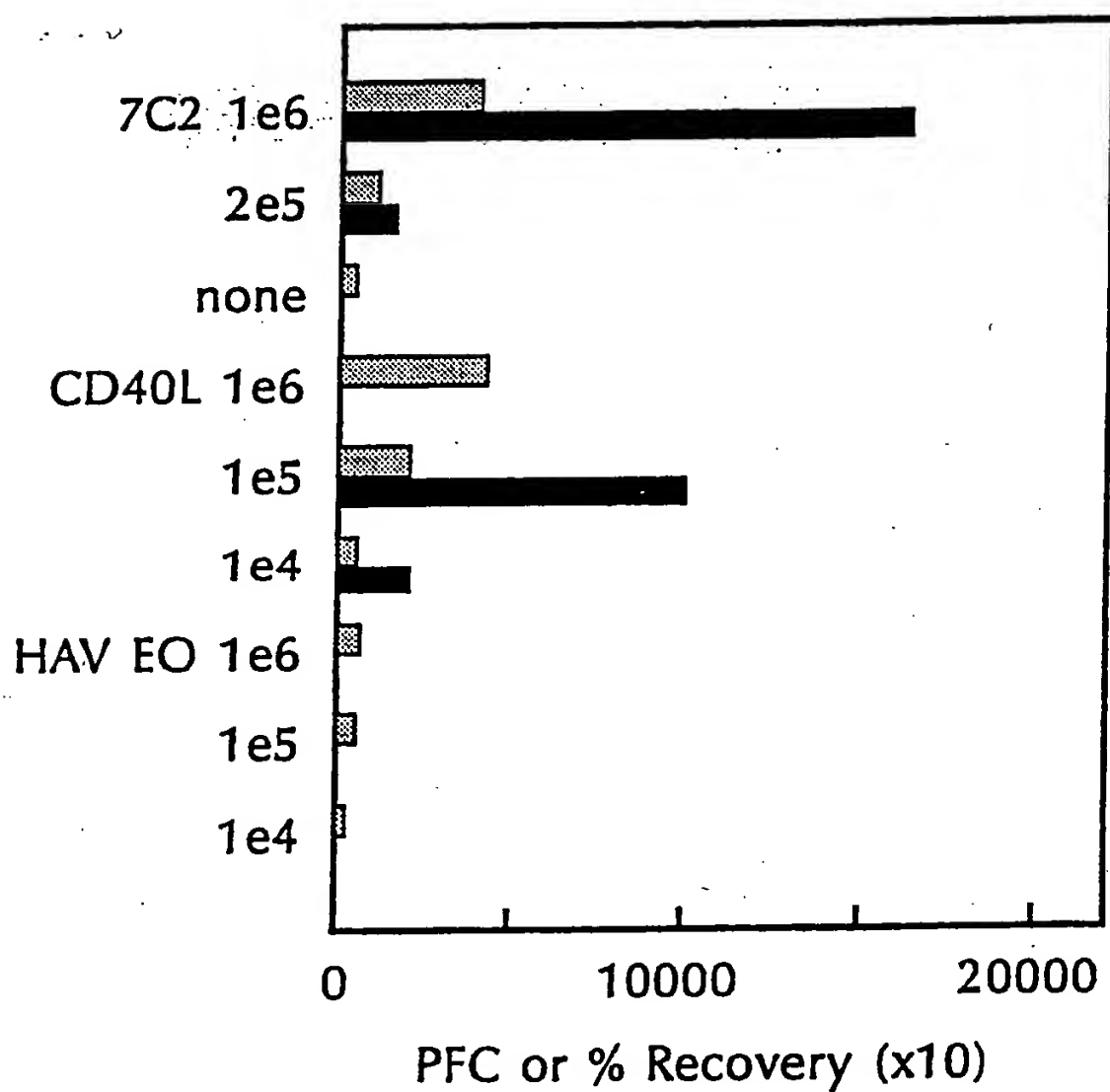
▨ % Recovery

■ PFC

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FIG. 12

Induction of Murine B Cell Proliferation by CD40 Ligand Expressing CV-1 Cels (fixed)
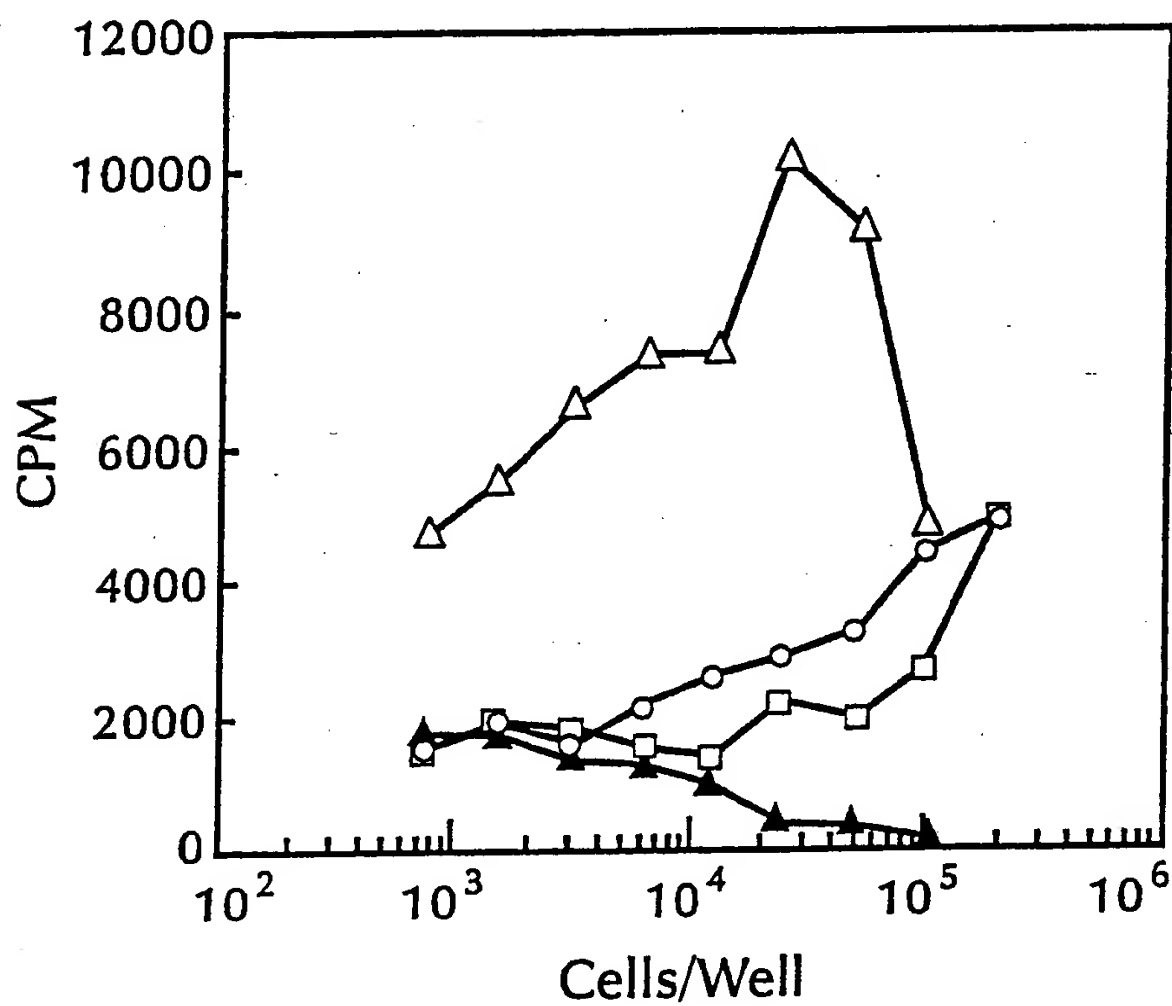
CD40L
HAV EO
7C2 11/6
7A1 11/6

FIG. 13A

Induction of Antigen Specific PFC by
CD40 Ligand Expressing CV-1 Cells (fixed)
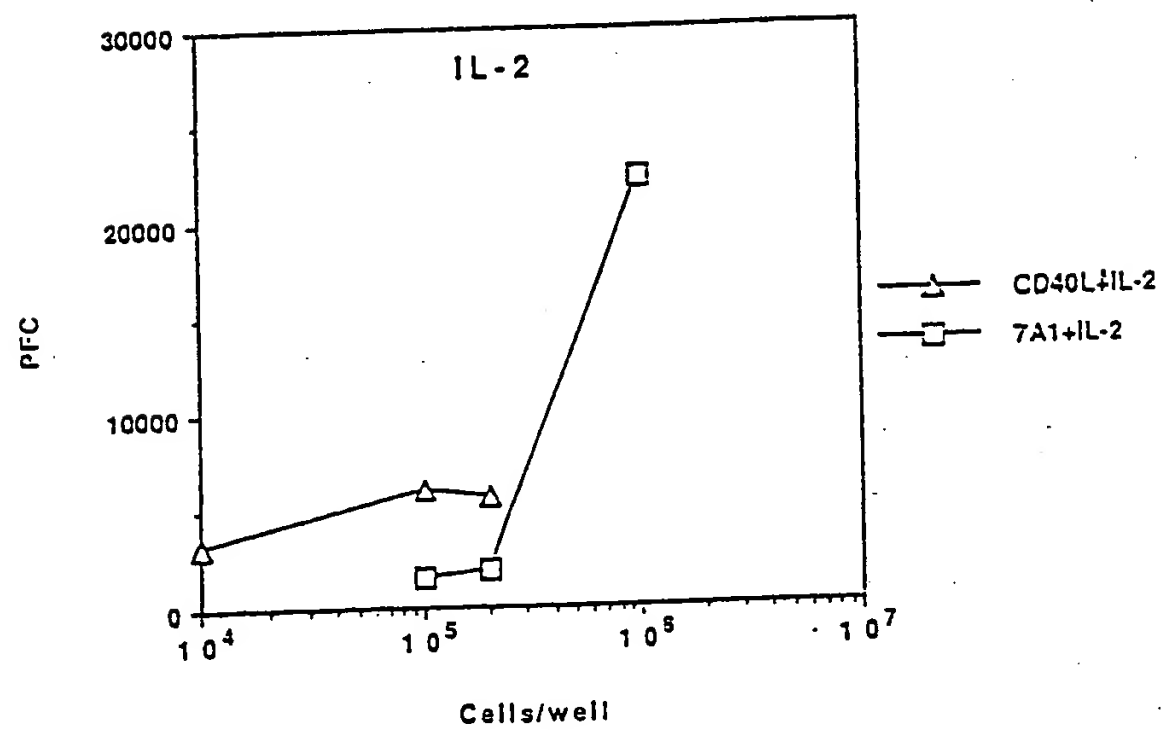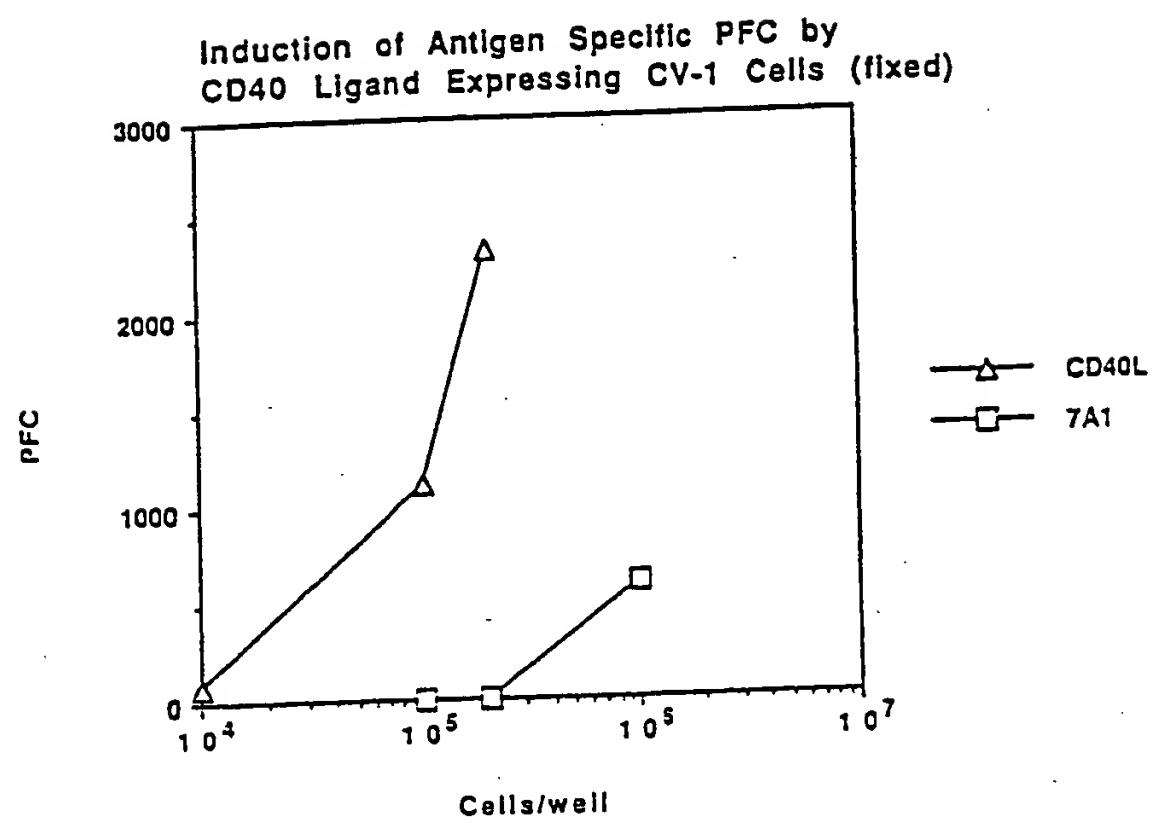
FIG. 13B

FIG. 14A

Day 7 Proliferation of T-depleted PBM

o HAV
● CD40L
△ CD40L + CD40.Fc
▲ CD40L + TNFR.Fc
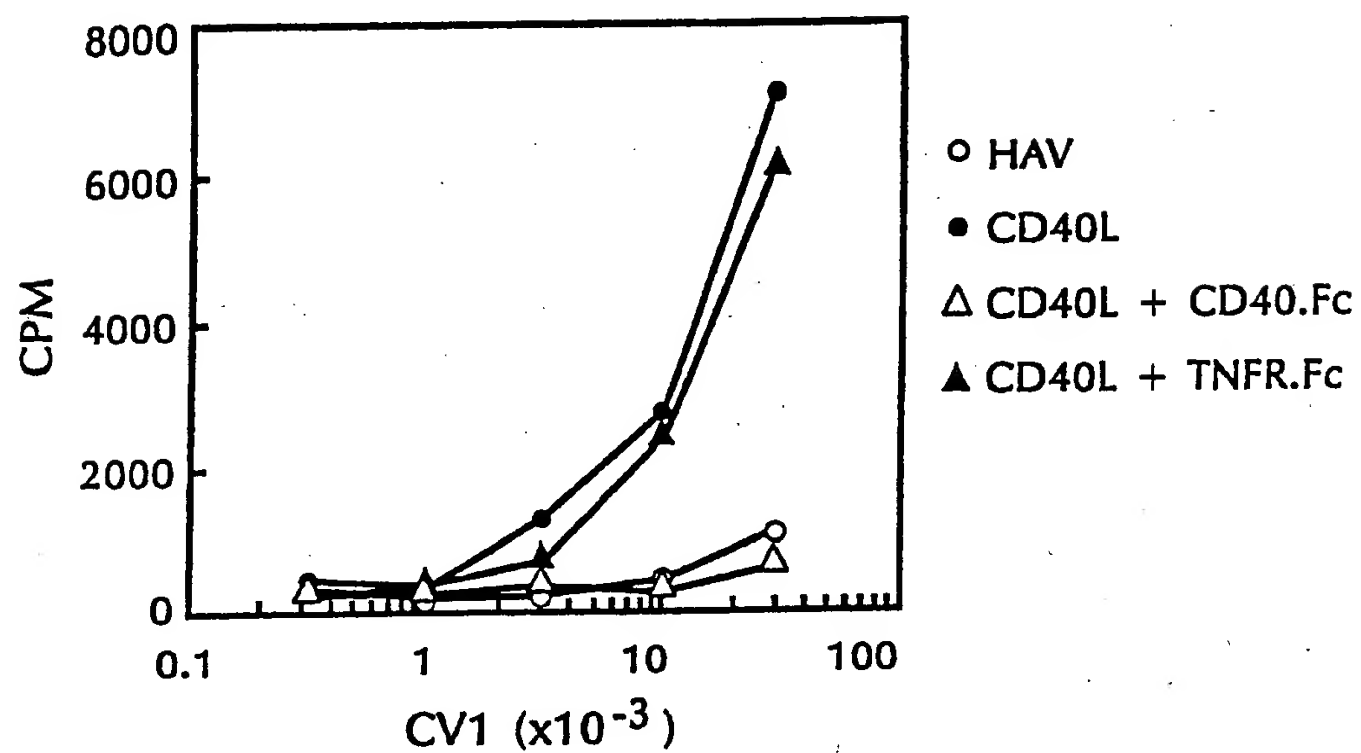
FIG. 14B

Day 10 IgE Secretion from T-depleted PBM

IL-4 alone   0
IL-4 + G28-5   29.7 ng/ml

☐ HAV
▨ CD40L
▨ CD40L + CD40.Fc
■ CD40L + TNFR.Fc
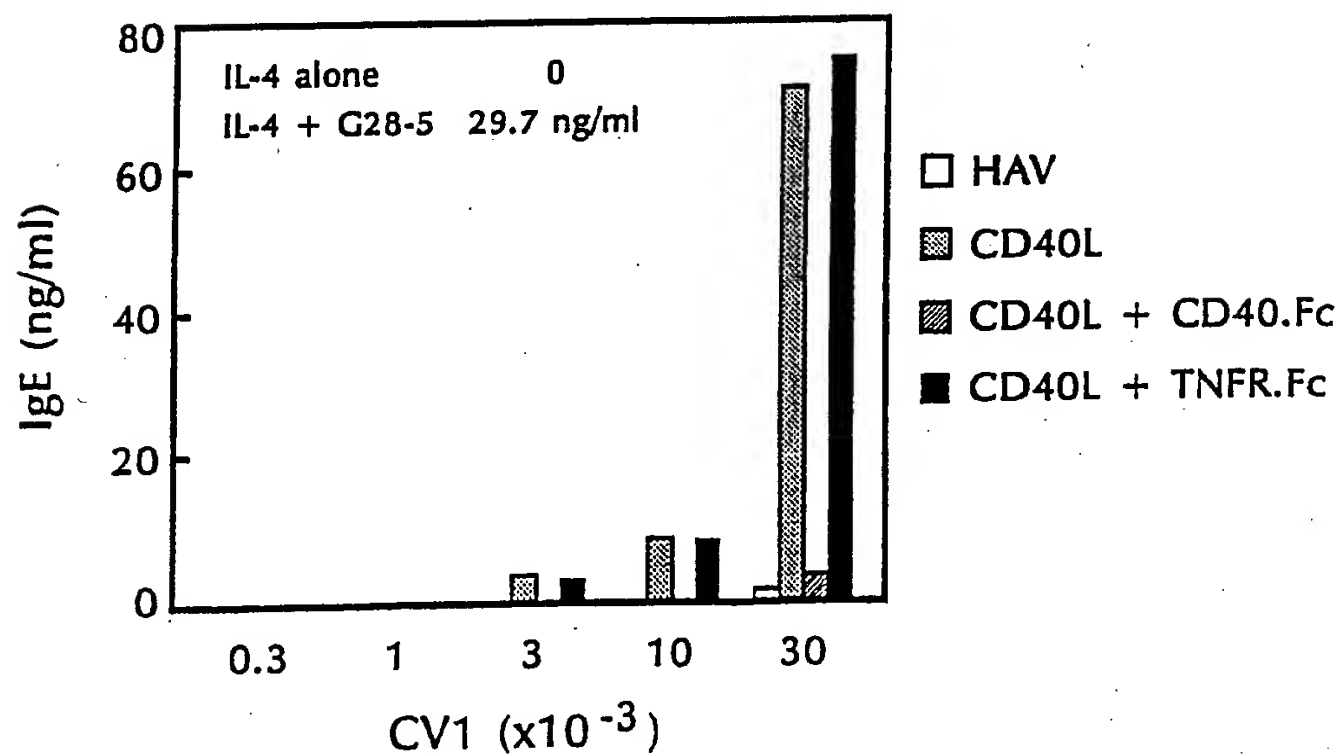
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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/27; 530/388.22, 388.23, 389.2, 351; 435/70.3, 69.1, 69.5, 240.1, 252.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

search terms: CD40, ligand, antibodies, recombinant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Nature, Volume 350, Issued 04 April 1991, M.L. Riordan et al., "Oligonucleotide-based therapeutics", pages 442-443, entire document.	20
Y	The EMBO Journal, Volume 8(5), issued 1989, I.Stamenkovic et al., "A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas", pages 1403-1410, entire document, especially page 1408, 2nd column, paragraphs 2-3.	1-14, 17-20
Y	Proceedings of the National Academy of Science, Vol. 84, issued December 1987, A. Aruffo and B. Seed, "Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system", pages 8573-8577, entire document, see especially page 8573, §2.	1-6

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

	Special categories of cited documents:
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"E"	earlier document published on or after the international filing date
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"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 January 1993	26 JAN 1993

| Name and mailing address of the ISA/
Commissioner of Patents and Trademarks
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Washington, D.C. 20231
Facsimile No. NOT APPLICABLE | Authorized officer

LORRAINE M. SPECTOR, PH.D.

Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C07H 21/00: A61K 35/14; C07K 3/00, 7/00, 13/00; C12P 21/02, 21/06; C12N 15/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

536/27; 530/388.22, 388.23, 389.2, 351; 435/70.3, 69.1, 69.5, 240.1, 252.3

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(74) Agent: PERKINS, Patricia, Anne; Immunex Corporation, 51 University Street, Seattle, WA 98101 (US).

(54) Title: CD40L MUTEIN

(57) Abstract

There is disclosed a polypeptide that is mutein of CD40L, and DNA sequences, vectors and transformed host cells useful in providing such CD40L muteins.

Inter onal Application No

PC /US 96/09632

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/12   C07K14/725   C12N15/62   C12N5/10   C12N1/19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C07K   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 08207 (IMMUNEX CORPORATION) 29 April 1993 cited in the application see the whole document ---	1,2
A	WO,A,94 10308 (IMMUNEX CORPORATION) 11 May 1994 see example 1 see page 4, line 20 - page 6, line 17 --- -/--	1-8

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 November 1996	1 1 12 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Le Cornec, N

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page 1 of 2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOCHEMISTRY, vol. 34, no. 6, February 1995, EASTON, PA US, pages 1833-1844, XP002019526 J. BAJORATH ET AL: "Identification of residues on CD40 and its ligand which are critical for the receptor-ligand interaction" see the whole document ---	1
A	EP,A,0 585 943 (BRISTOL-MYERS SQUIBB COMPANY) 9 March 1994 see page 6, line 26 - page 8, line 27 see page 11, line 1 - line 48 -----	1

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9308207	29-04-93	AU-B-	661360	20-07-95
		AU-A-	3122693	21-05-93
		CA-A-	2121798	29-04-93
		EP-A-	0667901	23-08-95
		FI-A-	941837	30-05-94
		JP-T-	7504083	11-05-95
		NO-A-	941422	27-06-94
WO-A-9410308	11-05-94	AU-A-	5537794	24-05-94
		CA-A-	2146559	11-05-94
		EP-A-	0672141	20-09-95
		JP-T-	8507676	20-08-96
		NZ-A-	257942	26-04-96
EP-A-585943	09-03-94	US-A-	5540926	30-07-96
		AU-A-	4612093	10-03-94
		CA-A-	2105552	05-03-94
		FI-A-	933862	05-03-94
		HU-A-	69977	28-09-95
		JP-A-	6315383	15-11-94
		NO-A-	933126	07-03-94
		NZ-A-	248569	26-10-95
		ZA-A-	9306491	25-03-94

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